Effect of pre-acclimation to sub lethal dose and combination of cold stress on tolerance of *Folsomia candida* to thiacloprid

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Abstract

Living organisms are usually exposed to combinations of a number of physical and chemical environmental factors in their natural habitats. Pre-exposure to sub lethal doses of a chemical may induce enhanced tolerance to higher doses of the chemical during successive exposures. Pre-exposure may also make organisms more sensitive to later exposures to smaller doses of the chemical. Toxicity of chemicals is also significantly affected by physical environmental variables. Temperature is among the most important physical environmental factor that interact with toxic chemical substances affecting their toxicity. Due to these interactions, results from controlled laboratory experiments that consider a single factor cannot represent the true effect of the toxicant on an organism in its natural environment. Hence, for better safety recommendations, environmental risk assessment of toxic chemicals should consider as much environmental factors as possible.

This study evaluates toxicity of thiacloprid to *F. candida* under two conditions. The first experiment evaluates toxicity of thiacloprid following pre-exposure (acclimation) to sub lethal concentration of the chemical. The second experiment assesses development of cross tolerance by *F. candida* to thiacloprid when combined with cold stress. In the pre-acclimation experiment, *F. candida* were exposed to sub lethal doses for two weeks before they are subject to higher doses. The cross tolerance experiments on the other hand evaluate toxicity of thiacloprid when combined with cold stress. The experiments were conducted by combining the two stress factors in three different orders (1. Cold and thiacloprid applied simultaneously, 2. Thiacloprid prior to cold, 3. Cold prior to thiacloprid).

Pre-exposure had significantly reduced range of tolerance of *F. candida* to thiacloprid. ANOVA showed that pre-exposure reduced the concentration that kill 50 % of the population by a factor of five. Reproduction was also significantly reduced following pre-exposure. On the other experiment, cold stress and thiacloprid did not show development of cross tolerance. However, results from simultaneous exposure indicate that cold temperatures reduced toxicity of thiacloprid. Acute LC50 of thiacloprid during simultaneous exposure was 7 times less at 7 ^{0}C than the LC50 at 15 ^{0}C. However, cold stress applied either prior or following to thiacloprid did not show any effect on toxicity of thiacloprid. Adverse effect of thiacolprid on reproduction of *F. candiad* was also significantly aggravated by pre-exposure and cold stress.

Introduction

Ecotoxicological studies of contaminants are difficult to conduct in a natural ecosystem due to the presence of many confounding factors. It is difficult to see the toxic effect of any given chemical independently under a complex system such as a whole ecosystem. Therefore, most studies of ecotoxicity depend on simple designed laboratory experiments with few factors of variation. However, laboratory results do not provide precise pictures of what a given toxicant does in nature where many other interacting environmental and climatic factors can significantly modify the effect (Holmstrup et al., 2008).

Terrestrial toxicity tests of chemicals are significantly affected by bioavailability of the chemical and adsorption and retention capacity of the soil. Toxicity is also affected by concentration of the chemical in the soil pore space. These features of the chemical in the soil are significantly affected by such important soil characteristics as organic matter content, pH, clay content and soil texture (Pedersen et al., 1997). Moreover, clay content and structure of the soil affects moisture content of the soil that have direct effect on the degree to which organisms can get exposed to the chemical in a short term.

It is practically impossible to include all species of an ecosystem for ecological studies. Therefore, toxicity tests are conducted based on some selected species that are representative of the ecosystem. Test species for ecotoxicological studies are selected based on their representativeness to the ecosystem which is in turn measured by extent of their relevance to ecological systems. Abundance and distribution over diverse ecological zones and relative sensitivity to contaminants (Diogo et al., 2007), as well as ease of reproduction in simple laboratory cultures and short generation time (Fountain and Hopkin, 2005; Broerse and van Gestel, 2010) are the main criteria in selecting candidate species for ecotoxicological tests. Springtails fulfill most of these criteria and hence are regarded as one of the common representative organisms for toxicity testing of the edaphic ecosystem. They have been used for various toxicity tests and for testing different ecological stress factors in the edaphic ecosystem (Pederson, et al., 1997; Crouau et al., 1999; Sjursen et al., 2001; Liefting et al., 2010; and Liu, et al., 2010).

Ecology of springtails

Springtails belong to the order Collembola under the phylum Arthropoda that contains the largest number of species diversity and abundance. Springtails are among the most abundant microarthropods living in a wide variety of soil ecosystems (Fountain and Hopkins, 2005; Styrishave, et al., 2010) that contain about 8000 different species known to science to date (Deharveng et al., 2007). High reproduction capacity that includes prolific egg laying, early hatching, short period to reach reproductive maturity and a life span with repeated reproductive potential are among the main factors that contribute to their abundance in their habitats under optimum conditions (Fountain and Hopkins, 2005).

Their small body size also enables them to survive in masses in small spaces with meager food resources. Butcher et al. (1972) mentioned in their review that it is possible to obtain up to 46,700 individuals of springtails in a square meter of soil under optimal conditions.

Springtails are well adapted to climatic stressors such as temperature and drought (Sjursen and Holmstrup, 2004). Even though they have very low active dispersal ability, their small size and minimal mass also enables them to disperse into large areas at faster rates by wind and other animals (Brand and Dunn, 1998). Their high dispersal combined with their quick adaptation to climatic factors help springtails to occupy varied habitats of different ecological characters (Fountain and Hopkins, 2005). They are reported to be among the very few organisms that contain species which survive even the very harsh environment of Antarctica (Hogg and Stevens, 2002; Slabber et al., 2007). Springtails normally inhabit the pore space of the soil (Fountain and Hopkins, 2005).

Although a few cases of predation have been observed in springtails (Chamberlain, et al., 2006), most of the species are herbivores or detritivores (Brand and Dunn, 1998). Fungi are the most preferred food for springtails (Klironomos et al., 1991; Sawahata et al., 2000), but bacteria, nematodes, plant leaves and other decaying organic matter (Sawahata et al., 2000; Chamberlain, et al., 2006) are also other recorded sources of food. They are also important prey animals for invertebrate predators such as mites, centipedes, spiders, carabidae and some beetles (Environment Canada (EC), 2007; Hogg and Stevens, 2002).

Due to their preference for organic detritus as food, springtails are not known to have direct significant impact as agricultural pests. However, they can serve as host organisms for some plant pathogens (Crossley et al., 1992; Rusek, 1998).They can also play important role as biocontrol agents against some bacterial or fungal plant pathogens, or as host animals for other species which feed on some plant pathogens (Rusek, 1998). Fountain and Hopkins (2005) reviewed that *F. candida* has been used to significantly reduce incidence of bacterial fire blight in fruit trees and fungal white mold in many plants by feeding on the bacteria and the fungal pathogens that cause the diseases.

Springtails are part of the mesofauna that play an important functional and structural role in the ecosystem, facilitating decomposition of organic matter and nutrient recycling (Rusek, 1998; Choi et al., 2006; Environment Canada, 2007; Fjellberg, 2010; Styrishave et al., 2010). On the other hand, they can positively or negatively affect decomposition by affecting population of fungal species depending on the springtail and fungal species involved (Tordoff et al., 2008). This arises mainly due to their preferred grazing on fungal species that are known as primary decomposers (Fjellberg, 2010). They can also facilitate decomposition indirectly by inducing secondary processing by other microorganisms (Kaneda and Kaneko, 2007). As indicated in one study, overgrazing by populations of *F. candida* severely declined the fungal biomass that resulted in reduced rate of

decomposition (Tordoff et al., 2008). This could also cause other structural and functional impacts as it can affect the soil structure and availability of nutrients for other components of the saprophytic community. Feeding behavior of different species of springtails can affect carbon and nutrient availability by influencing microbial and nematode activity (Kaneda and Kaneko, 2007). Thus, managing populations of springtails has higher ecological relevance.

Folsomia candida

Folsomia candida was first described in 1902 by Willem (Fountain and Hopkins, 2005). It is a white species measuring a length of 1.5-3.0 mm at maturity. It is the springtail species most widely used as a test organism in terrestrial ecotoxicology risk assessment tests (Fountain and Hopkins, 2005; Slotsbo, et al., 2009; Liu et al., 2010). As common to all springtails, *F. candida* has a pair of structures called ventral tube or collophore that are lined together in close proximity on the ventral side of the first abdominal segment. These are thin-walled vesicular structures that can be turned from inside out. The thin lining enables these structures to serve as media of fluid exchange with the external environment. Therefore, they also serve as important routes of exposure to contaminants (Fountain and Hopkins, 2005).

F. candida has a very high reproductive capacity. Under optimal conditions in the laboratory, the species reaches reproductive maturity within three weeks of age (Fountain and Hopkins, 2005). As observed from our laboratory cultures, however, egg laying capacity declines at older ages. Usually massive egg laying is induced by transfer to new cultivation plaster plates. However, this has very little effect in older cultures (personal observation). Using synchronized culture of known age is therefore essential to reduce variability.

Eggs are deposited in numerous batches and also in single. The eggs are yellowish when they are fresh and turn to golden color at maturity. Eggs do not tolerate desiccation or too moist conditions. Time of hatching depends on the temperature. As has been observed in our laboratory cultures and also reported by Fountain and Hopkins (2005), hatching occurs between 7-10 days at 20 ^{0}C.

Duration of developmental stages and life cycle varies depending on temperature and other limiting factors such as food. At 20 ^{0}C one life cycle takes about 4-5 weeks. Due to their high fecundity they are capable of colonizing and filling their niche quickly. Reproduction in *F. candida* occurs exclusively by parthenogenesis (Fountain and Hopkins, 2005).

The range of optimum temperature at which *F. candida* attain higher fecundity, short reproduction time and faster development is 5 °C - 20 °C (Sjursen and Holmstrup, 2004). A study on cold survival of *F. candida* showed 20-40 % percent survival at 0 °C (Bayley et al.,

2001). However, another study recorded 100 % survival of the species at 0 °C (Holmstrup, Aubail et al, 2008). Moreover, these authors reported that the species is well adapted to freezing temperatures below that reaches -5.5 °C (Holmstrup et al., 2008).

Generally, mechanisms of cold tolerance in invertebrates can be divided into freeze avoidance and freeze tolerance (Bahrndorff et al., 2009). Like all other collembolans *F. candida* use freeze avoidance strategy (Bahrndorff et al., 2009). The two main physiological strategies involved in freeze avoidance are supercooling (Sinclair and Sjursen, 2001; Bahrndorff et al., 2009; Holmstrup et al, 2010) and production and accumulation of sugars and polyols which are referred to as cryoprotectors (Bahrndorff et al., 2009). By supercooling mechanism springtails dehydrate their body content (Sinclair and Sjursen, 2001; Holmstrup et al., 2010) and stop ingesting any food particle in addition to emptying the food particles from their gut (Bahrndorff et al., 2009) so that the freezing point of their body fluid remains below the temperature of the external environment.

Freeze avoidance may also involve adjustment of membrane phospholipids (Holmstrup et al, 2002) which is achieved by desaturation of fatty acids of the membrane (Hazel and Williams, 1990). The composition of phospholipids is essential for membrane fluidity and associated properties. Cold exposed *F. candida* also showed changes in fatty acid composition of the phospholipid of their membrane (Bayley et al., 2001)Collembolans also empty their gut content and stop ingesting any food particle in response to cold exposure in order to prevent freezing (Hogg and Stevens, 2002).

Ecotoxicology

Occurrence of springtails in varied habitats of different ecological systems gives toxicology research on springtails great ecological relevance. Even though various other species are also used for the same purpose, *F. candida* is the species that is most frequently used for studies of ecotoxicity. This species is considered by the International Standards Organization (ISO) as a standard arthropod species for soil toxicity testing (Krogh, 2009).

Fountain and Hopkins (2005) mentioned different ways of exposing *F. candida* to contaminants. Exposure through contaminated food particles underestimate results of toxicity as the springtails avoids the contaminated food after tasting it. According to Fountain and Hopkins (2005), the most common route of exposure that is applied by ISO standard test is through contact with contaminated pore water in the soil.

Magnitude of toxic effects in an organism depends on the concentration of the toxicant absorbed in the body, which in turn depends on the bioavailability of the chemical. Bioavailability refers to the form of a chemical in an environment which can be readily assimilated by living organisms (Wright and Welbourn, 2008). It is determined by the proportion of the chemical that is not bound to the soil particles (Sjursen and Holmstrup,

2004). This feature of a chemical in a soil is affected by organic matter content, pH and soil texture of the soil (Crommentuijn et al., 1997; Styrishave et al., 2010). Moisture is also an important parameter affecting exposure of springtails to chemical contaminants. Springtails normally occupy the air-filled pore space of the soil and avoid full contact with soil water (Pedersen et al., 1996). The soil should, therefore, be gently moist just enough to fill about 50 % of the soil pore space in order to get springtails well exposed to chemical contaminants (Fountain and Hopkins, 2005). The volume of water enough to keep the soil gently moist varies with texture of the soil. Therefore, it is important to define the soil type where toxicity tests are conducted. Organisms try to respond to environmental stressors after exposure by adjusting their physiology or behavior. However, organisms may be predisposed for other types of environmental stressor while adjusting their physiology in response to one environmental factor. For example, pre-acclimation to drought caused desaturation of membrane phospholipid fatty acid in *F. candida*, which resulted in higher susceptibility of the species to heat exposure (Holmstrup et al., 2002). Exposure to mercury was also found have an adverse effect on heat tolerance of springtails by destabilizing membrane function possibly by the action of the reactive oxygen species released by metabolism of mercury (Slotsbo et al., 2009). Therefore, it is important to understand the interaction of environmental factors in and outside the body.

Generally, environmental factors do not act independently under natural conditions. Climatic factors usually accompany effects of chemical pollutants in the environment. Climate may modify the effects of chemical contaminants, and may result in antagonistic or synergetic effects. Few studies have investigated synergetic effects of climatic factors and chemical pollutants. Sjursen et al (2001) reported synergetic effect of some polycyclic aromatic compounds and drought on reproduction and survival of *F. fimetaria*. On the other hand, Sjursen and Holmstrup (2004) found that drought exposure induced tolerance to pyrene in springtails.

Temperature is one of the most important climatic factors that interact with the chemical contaminants. It is also the most important environmental factor that continuously varies determining ability of organisms to adapt to chemical contaminants (Bendarska, et al., 2009). It affects toxicity of chemicals both before and after absorption inside the body. Since temperature influences kinetics of all biochemical processes, it affects bioavailability, absorption and transformation to toxic forms of contaminants and rate of its delivery to target sites (Martikainen and Rantalainen, 1998). It can modify toxicity directly by acting on the chemical or indirectly by changing physiology of the organism in a way that may increase tolerance or vulnerability (Bednarska et al., 2009). Various authors have demonstrated synergetic effects of temperature and chemical toxicants (Holmstrup et al., 2008; De Boer, et al., 2010).

Effect of temperature is more pronounced in ectothermic organisms such as arthropods (Martikainen and Rantalainen, 1998). Due to temperature-induced fluctuations of metabolism of poikilothermic organisms, toxicity of chemicals is affected by temperature. According to these authors, slower metabolic rates under colder temperatures reduce the rate of toxicity of contaminants. Higher temperatures, on the other hand, are reported to accelerate metabolic rates, thereby facilitating toxic transformation of contaminants. On the other hand, the authors report that higher temperatures may also facilitate excretion of chemicals following faster detoxification. These explanations suggest that the fate of chemicals in the face of variable temperature and other environmental variables depends on the inherent nature of the chemical, its interactions with the environmental factors and physiology of the organism (Martikainen and Rantalainen, 1998; Bednarska et al., 2009).

In most cases climatic factors interact with toxic chemicals synergistically (Sjursen et al., 2002). Cold stress and mercury have shown synergetic effect on survival of *F. candida* (Holmstrup et al., 2008). Sjursen and Holmstrup (2004) also found synergetic decrease in survival of *F.candida* as a result of exposure to drought and pyrene. In some instances, cross tolerance to chemical contaminants may develop as a result of exposure to sub lethal climatic stress. Sjursen and Holmstrup (2004) found cross tolerance in *F.candida* to pyrene due to prior exposure to cold stress. Pre-exposure to lower doses of a chemical (pre-acclimation) may induce tolerance to subsequent lethal doses. Valandoost (1999) reported a high level of enhanced tolerance in mosquito to permethrin as a result of pre-exposure to a sub lethal dose. He suggested various mechanisms of developing tolerance including genetic, enzymatic and behavioral changes following pre-exposure to the sub lethal dose.

As it has been discussed above, Change in membranes structure due to cold exposure may also have special importance regarding molecular toxicity of chemical contaminants that may follow or accompany cold exposure. Usually chemicals need to cross the membrane in order to cause their toxicity at the molecular level. Therefore, any factor that may have effect on membrane structure or integrity may have indirect effect on actual toxicity of chemical contaminants.

To provide reliable environmental safety recommendations, ecological risk assessment of chemical contaminants should consider as many combinations of risk factors as possible. Pesticide application does not usually subject all organisms in target are to equal amount of the dose applied. There are some members of target and non-target organisms that receive sub lethal doses from drift of pesticide application. This may contribute to the development of tolerance in those organisms that receive sub lethal doses (Valandosst, 1999). Moreover, physical environmental factors can change the effect of chemicals on organisms. Effects of sub lethal doses are assessed by conductiong pre-acclimation experiments. So far, no study has reported on effects of pre-acclimation and combination of cold stress toxicity of thiacloprid in terrestrial ecosystems. This study is therefore initiated to assess

environmental impact of thiacloprid under different environmental situations: pre-exposure and in combination with cold stress. The study was initiated with the following three hypotheses:

1. There is no effect of pre-exposure on sensitivity of *F. candida* to thiacloprid.
2. There is no effect of cold stress, either applied as pre-treatment, subsequent to or simultaneously with thiacloprid.
3. There is no effect of thiacloprid on cold tolerance of *F. candida*.

Based on these hypotheses the study is designed to investigate:

- Effect of pre-exposure to lower sub lethal dose on toxic tolerance of *F. candid* to subsequent higher concentrations of thiacloprid.
- Response of *F. candida* to combination of thiacloprid and cold stress

Materials and methods

Test species and preparation of synchronized culture

The test species *F. candida* W (Collembola: Isotomidae) was obtained from a permanent culture at the Institute of Biology, University of Oslo. Synchronized culture of the species was prepared to get adults of the same age to be used for the experiments. Culturing was made on a plate in a petridish made of moistened Plaster of Paris and activated charcoal. The cultures were kept in 20 ^{0}C and fed with dry yeast and moistened by adding few drops of distilled water every week. Any yeast grain left every week was removed to avoid fungal contamination of the plates, and new food was added.

Preparation of the synchronized culture was started by inducing the adults obtained from the cultures for egg laying by transferring them to new culture plates. Egg batches were collected and kept in a separate new plate every two days for about one week. The eggs were followed up for hatching every three days and hatching time was marked on the petridishes so that adults of 5-6 weeks old will be identified to be used for the experiments.

Experimental Soil type

The soil type used was sandy light clay which has clay content of 7 %. The soil was obtained from experimental fields of Life Science University (UMB) at Ås. Organic carbon content of the soil was 1.1 %. Other physical and chemical properties of the soil are presented in table 2.1. The soil was sterilized by burning at 600 ^{0}C for 5 hours.

Experiments with springtails should keep the water moist only to the level it fills the water pore space of the soil. This has been decided for our experiment soil subjectively by touching the texture of the moist soil. Thus it was kept moist with distilled water equivalent in volume to 20 % of mass of the soil.

Test chemical and preparation of test concentrations

The test chemical thiacloprid is produced by Bayer CropScience as a commercial formulation known as Calypso. It is formulated as suspension concentrate and the active ingredient, thiacloprid, constitutes 48 % of the formulation. It is known by a generic name [3-[(6-chloro-3-pyridinyl)methyl]-2-thiazolidinylidene]cyanamide. It is a chloronicotinoid class of chemical applied for non restricted use. It exerts its toxic effect by inhibiting acetylcholine receptors leading to disruption of the nervous system (United States Environmental Protection Agency (USEPA), 2003). Moreover, the technical note reported that it is stable under elevated temperature and presence of metals and metal ions ensuring its toxic potential under these various circumstances. Under aerobic condition toxicity of thiacloprid can persist up to one month (Wang et al., 2008).

Range finding test was conducted in order to determine the test concentrations for the main experiment set ups. The range finding test was conducted in two steps each including five test concentrations with one untreated control. Each successive dose set by doubling the previous dose. The first test included wider range taking 125 mg/kg as the lowest dose and 2000 mg/kg as the highest dose. Based on the survival data that proved all doses caused more than 50 % mortality, the range of the test concentrations in the second step was narrowed to 200 mg/kg. Hence the lowest and the highest doses were 12.5 mg/kg and 200 mg/kg, respectively. Based on the results of the range finding test that identified 125 mg/kg as the LC50, three sub-lethal concentrations were chosen to be the test concentrations. The test concentrations were determined to be multiple of five so that the next higher dose was chosen by multiplying the lower dose by five. According to the LC50 values from the range finding tests, all the test concentrations were below the LC50. The entire set of test concentrations used in the pre-acclimation experiment and the three cross tolerance experiments were the untreated control (0 mg/kg), 5 mg/kg, 25 mg/kg and 125 mg/kg. Besides 5 mg/kg was the dose used as pre-exposure dose in the pre-acclimation experiment.

The experiment was conducted on 30 gm of soil spiked with the test concentration of thiacloprid diluted with 9 ml of distilled water that is equivalent to 30 % of the mass of the soil per replication. A stock solution of thiacloprid was prepared to avoid repeated measurement of each test concentration and associated source of errors. The stock solution was prepared by measuring double of the highest dose with four replications. Half of this was used to prepare the highest test concentration by diluting it with appropriate volume of distilled water (36 ml since it was prepared together for the four replications). The remaining half of the stock solution was used to prepare each lower dose by dilution series. In the case of the range finding test, each lower dose was prepared by diluting the dose just above by adding distilled that was twice the volume required to be applied to the soil since every successive dose was double to the lower dose. Whereas the test concentrations for

the next lower dose used in the main set of experiments was prepared by adding volume of distilled water five times what was actually required for the soil. Replications were divided after the soil was spiked by mixing it thoroughly with some volume of the stock solution that was equivalent to the intended test concentration.

Table 2.1: Physical and chemical properties of the soil used for the experiment

Chemical and physical properties	Unit of measurement	Measurement
pH	-	7.1
Sand	%	72.4
Silt	%	17.5
Clay	%	10.1
Cation exchange capacity	mmol (+)/kg	132
Percent base saturation	%	100
Excahngable acidity	mmol (+)/kg	<0.1
Organic carbon	g/100g TS	1.1
Total nitrogen	g/100g TS	o.13
Ammonium-N	mg/kg	4.5
Phosphorus	mg/kg	1150
Potassium	mg/kg	1210
Calcium	mg/kg	3120
Magnesium	mg/kg	2720
Sodium	mg/kg	83.6
Iron	mg/kg	16500
Sulfur	mg/kg	168
Aluminium	mg/kg	10500
Manganese	mg/kg	617
Zink	mg/kg	85.5
Copper	mg/kg	11.3
Vanadium	mg/kg	29.9
Titanium	mg/kg	406
Cobalt	mg/kg	5.6
Barium	mg/kg	68.7
Molybdenum	mg/kg	1.9
Nickel	mg/kg	18.8
Cadmium	mg/kg	0.19
Lead	mg/kg	13.9
Chromium	mg/kg	18.7

The study comprised four different sets of experiments. The first experiment tried to assess effect of pre-exposure to sub-lethal doses on general toxic effect of thiacloprid (Pre-acclimation). The remaining three experiments were designed to assess combined effect of cold exposure and thiacloprid as they happen simultaneously and in two sequential orders

there by evaluating development of cross tolerance for these stress factors. The cold exposure included two levels of low temperatures (0 ^{0}C and 7 ^{0}C) with a control at 15 ^{0}C.

Acute and chronic effects of pre-exposure to thiacloprid

In this experiment about 300 adults of 5-6 weeks old were pre-exposed to 5 mg/kg thiacloprid spiked with 300 gm of soil. Whereas another 300 adults of the same age were maintained in 300 gm of soil moistened only with distilled water as non-pre-exposed group (control group). The pre-exposure stayed for two weeks which was then followed by exposure of the two groups (pre-exposed and control groups) independently to the three main test concentrations with one unexposed control at four replications for two weeks. Effects were assessed by observing response on reproduction, movement and survival of the species at the end of the experiment period.

Reproduction was assessed as number of juveniles produced per single adult. Effect on movement was assessed from observation of immobilized individuals. Survival was measured by direct count of individuals with coordinated walking behaviour immediately at the end of the exposure period (and including the immobilized ones that displayed coordinated walking behaviour after 3 days). Data on survival and movement were analyzed as percentages of individuals actively moving and immobilized, respectively, as observed at the end of the exposure period. This was then recorded as percent score of the initial population. Immobile springtails were recorded as stunned individuals and transferred to a clean culture petridish and kept at 15 ^{0}C for one week to observe any movement. Moreover, microscopic observation was made to see any sluggish movement of appendages to identify stunned from dead individuals. Individuals that showed coordinated crawling were counted as immobilized (but surviving), otherwise they were considered as dead.

Cross tolerance

The cross tolerance experiments were conducted by combining the two stress factors in three different ways: 1. Exposure to cold stress and thiacloprid simultaneously, 2. Exposure to thiacloprid prior to cold stress, and 3. Exposure to cold stress prior to thiacloprid. Cold exposure was made on a clean plate made of plaster of Paris and activated charcoal prepared in a small box. The combined treatments of cold stress and thiacloprid were set at the beginning of the experiments so that they will be directly transferred to the pre-determined treatment level in the subsequent test or exposure. The treatments were replicated four times. In each set of experiment ten individuals of *F. candida* of 4-5 weeks old were used per test concentration per replication. In experiment 2, *F. candida* were exposed to the different test concentrations of thiacloprid at 15 °C prior to cold exposure. Duration for exposure period for each stress factor was two weeks.

Data on survival and reproduction (number of eggs and juveniles) were taken at the end of each exposure period. Survival was recorded twice (immediately and one week after the end of the exposure period). Floatation technique was used to take survival data as well as to transfer the surviving individuals to clean plates with spatula. In case of experiment 1 and experiment 3, they were kept in the clean cultures for recovery at 15 ^{0}C for one week when the second survival data as well as reproduction data were taken. In experiment 2, the surviving adults were transferred to the cold stress exposure in the clean plates for two weeks. After two weeks of cold exposure following the pesticide, they were kept at normal temperature for one week of recovery which ended by taking the second survival data and data on reproduction. Percentage difference of survival calculated from the two records of survival was used to determine the delayed effects of the pesticide. Egg laying was assessed during one week (in experiment 2) and two weeks (in experiments 1 and 3) after transferring the springtails to small boxes of clean plates.

Experiment 3 was made by first exposing *F. candida* to cold stress at temperatures 0 ^{0}C, 7 ^{0}C and 15 ^{0}C for two weeks. At the end of this period data on survival and reproduction were collected and the surviving adults were exposed to the three different concentrations of thiacloprid with one untreated control and kept at 15 ^{0}C for two weeks. Data on survival and reproduction were also taken at the end of this exposure period.

In experiment 3, ten individuals of *F. candida* were exposed to cold for two weeks prior to thiacloprid. After two weeks of cold exposure, data on survival and reproduction were recorded immediately before the springtails were transferred to clean boxes with culture plates and kept at 15 °C for 1 week for recovery so that survival data was taken again. Then the surviving individuals from each replication of the cold and thiacloprid treatments were subject for the subsequent exposure to thiacloprid for two weeks.

Data were taken three times for each experiment of cross tolerance. The first two data were taken at the end of exposure period to each stress factor. The last data were taken after recovery period.

Statistical analysis

Data was analysed using the statistical software R (R Development Core Team, 2010). For determination of the range finding test, data were transformed to logarithmic scale before they were subject for analysis. After the analysis they were back transformed.

The data from all sets of experiments were subject for two-way analysis of variance (ANOVA) by fitting the data to a generalized linear mixed effect model (GLMM). Parameters that showed significant variation were further sorted by doing mean separations with TukeyHSD.

Results

Range finding test

The LC 50 values predicted by the two range finding tests are indicated in table 3.1 and figures 3.1 and 3.2. The two tests did not show consistency for the predicted LC50 values. The first test estimated the LC50 at 195.42 mg/kg, which falls within the range of the test concentrations used. In the second test, the LC50 was estimated at 825.99 mg/kg, which is much higher than the highest tested concentration (200 mg/kg). Therefore, based on the results of the range finding tests, the subsequent main experiments of pre-acclimation and cross tolerance involve only sub lethal doses.

Effects of pre-exposure on survival and movement

ANOVA clearly indicated significant effects of pre-exposure on survival ($F=35.061$; $P<0.001$, Fig 3.5) and movement ($F=10.267$; $P<0.005$, Fig. 3.8). Highly significant reduction in survival and movement was observed from control populations in the pre-exposed groups compared to control populations that were not pre-exposed. Response in survival followed the same trend along doses of thiacloprid in the pre-exposed and unexposed groups (Fig 3.7), confirmed by differences being non-significant (ANOVA, 5 % significance level) among all test concentrations for differences in survival between pre-exposed and non-pre-exposed groups *F. candida* (Fig 3.6).

Survival follows a sigmoid response curve under both conditions (Fig 3.7). The highest effect was observed between 5 mg/kg and 25 mg/kg. However, no significant difference on survival was recorded between untreated replicates and those exposed to 5 mg/kg in the pre-exposed groups. Pre-exposure also lowered the LC 50 to 384.79 mg/kg which was estimated at 23370.5 mg/kg without pre-exposure (Table 3.1). However, ANOVA for the

Table 3.1: LC50 values the range finding tests and the pre-acclimation and cross tolerance experiments

Experiment/Test	LC50 values (Mean±SE)
Range finding 1	195.42 ± 1.07
Range finding 2	825.99 ± 1.4
Pre-acclimation	
- With pre-exposure	384.79 ± 1.72
- Without pre-exposure	23370.5 ± 3.76
Cross tolerance	
- ***Simultaneous exposure***	
a. At normal temperature (15 ^{0}C)	9.81 ± 1.07
b. At 7 ^{0}C	72.24 ± 1.11
- ***Thiacloprid prior to cold stress***	
a. At 15 ^{0}C	10.00 ± 1.06

b.	At 7^{0}C	7.49 ± 1.06
-	***Cold stress prior to thiacloprid***	
a.	At 15 ^{0}C	18.14 ± 1.08
b.	At 7 ^{0}C	10.36 ± 1.08

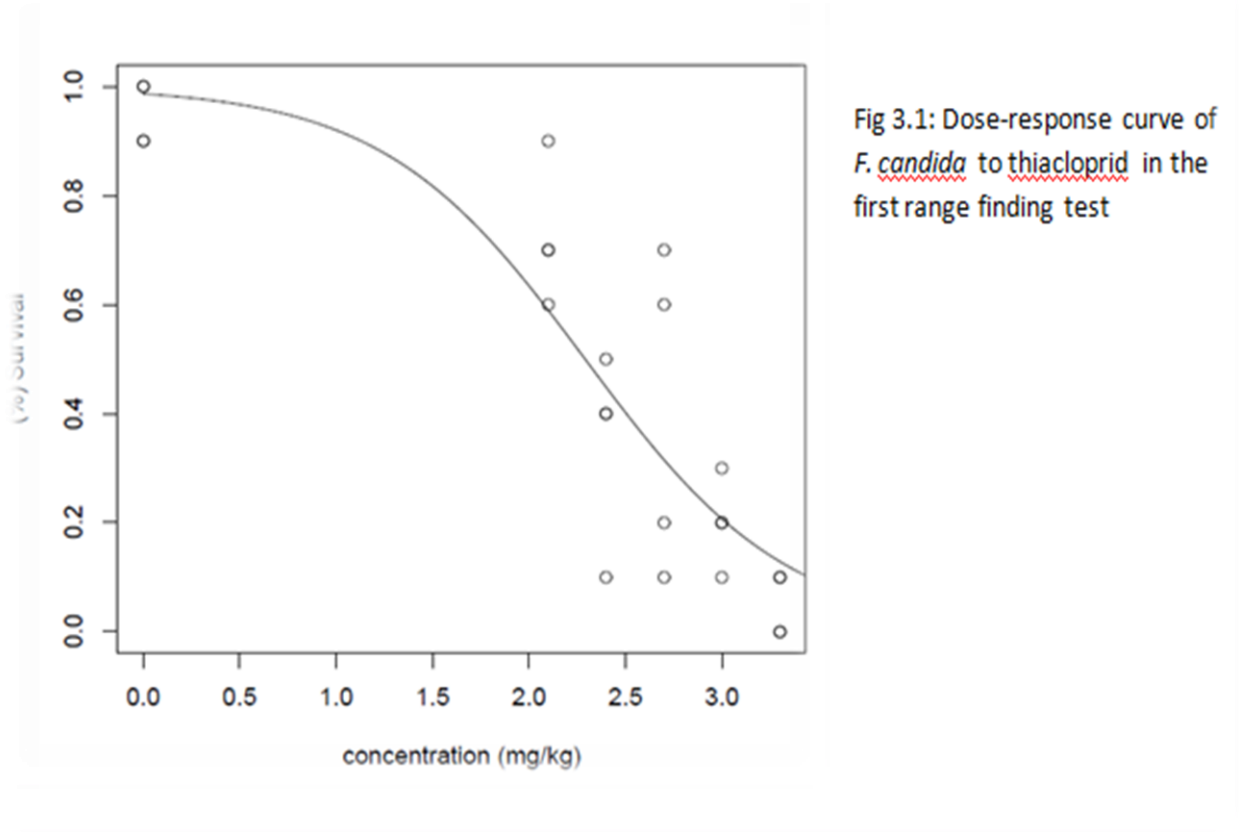

Fig 3.1: Dose-response curve of *F. candida* to thiacloprid in the first range finding test

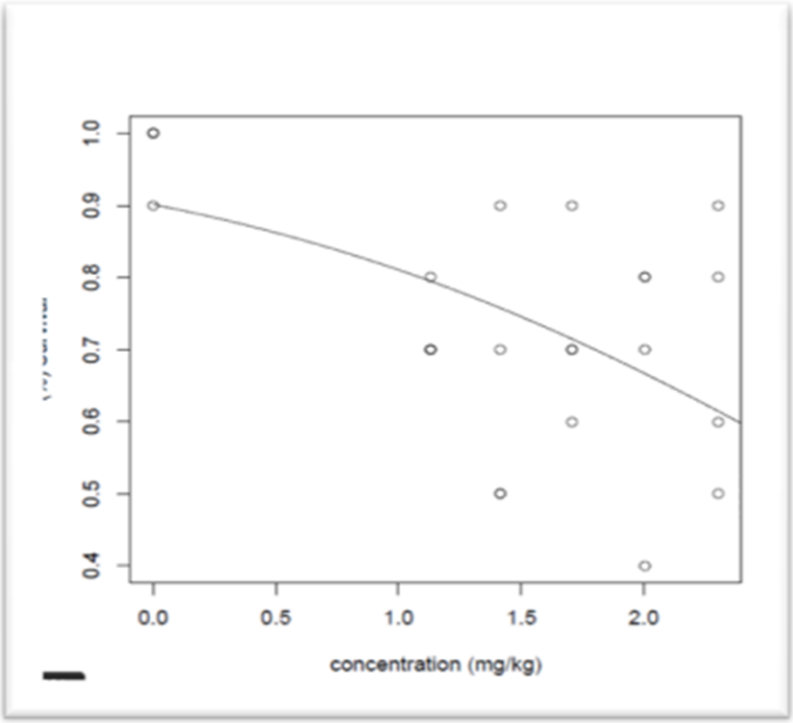

Fig 3.2: Dose-Response curve of *F.candida* for different doses of thiacloprid in the second range finding test

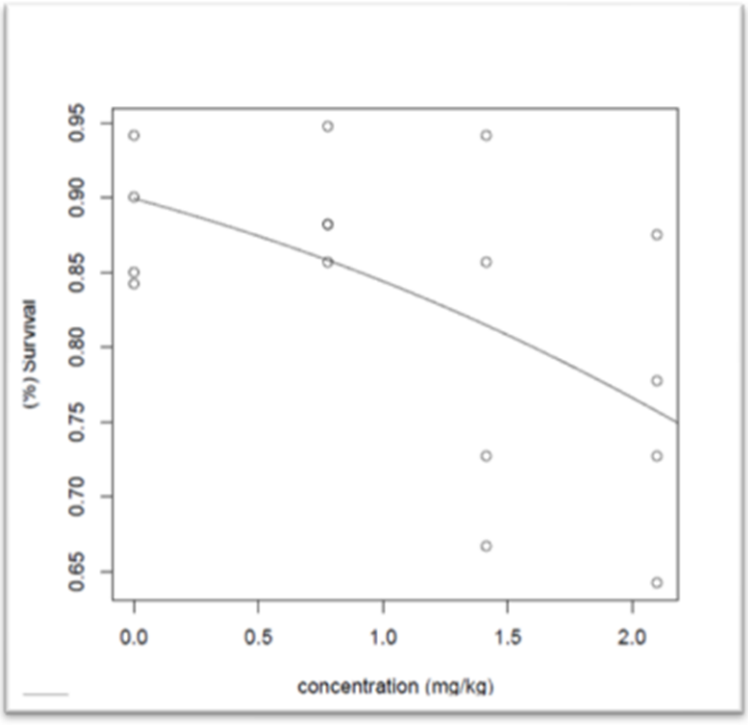

Fig 3.3: Dose-Response curve of *F. candida* to estimate the LC50 of not pre-exposed groups in the pre-acclimation experimet

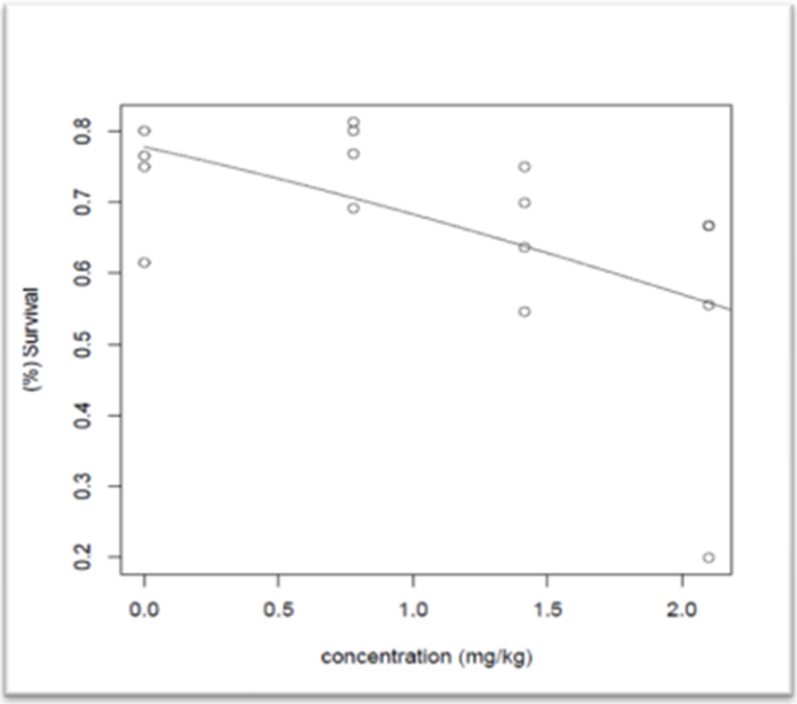

Fig 3.4: Dose-Response curve of *F. candida* to estimate the LC50 of not pre-exposed groups in the pre-acclimation experimet

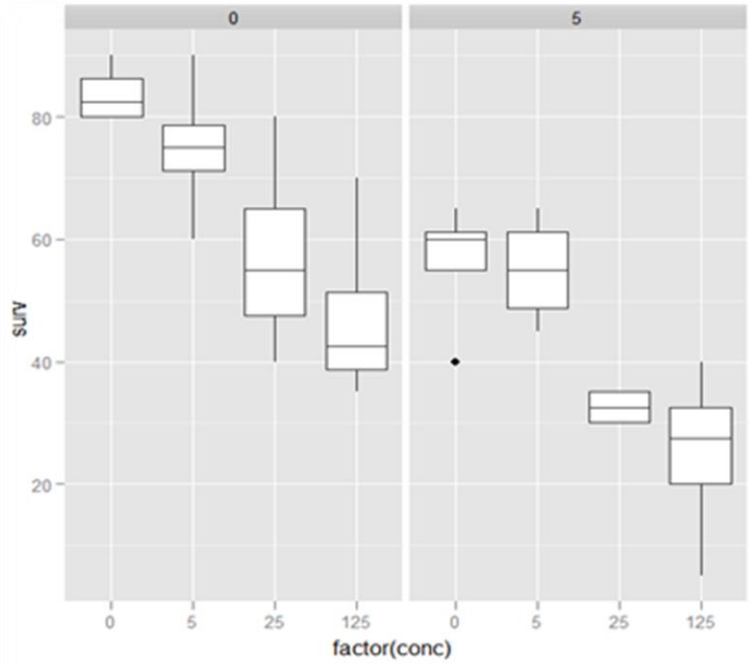

Fig 3.5: Survival (%) of *F. candida* after exposure to different concentrations of thiacloprid (mg/kg) with and without pre-exposure.

NB: Numbers o and 5 at the top of the two sides of the graph represent for the levels of pre-exposure (i.e. 0 mg/kg, groups control and 5 mg/kg)

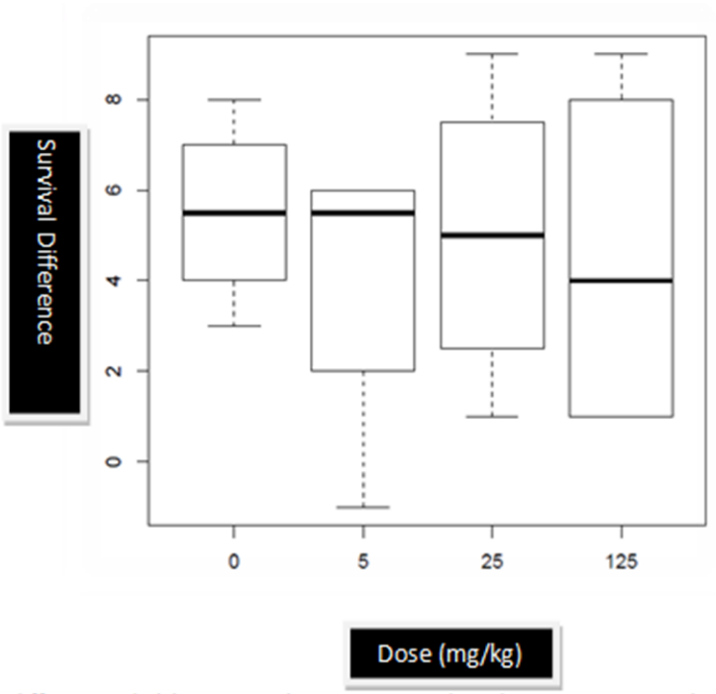

Fig 3.6: Survival difference (%) between the pre-exposed and non-pre-exposed groups of *F. candida* at different doses of thiacloprid

dose-response relationship for the groups without pre-exposure indicate that approximately 50 % mortality was recorded at 25 mg/kg (Fig 3.5). Responses were sharp for doses between 5 mg/kg and 25 mg/kg in both groups. The greatest effect of pre-exposure was observed at 5 mg/kg.

Movement was significantly affected by pre-exposure as explained by significant difference in proportion of immobilized individuals ($F=10.267$; $P<0.005$) between pre-exposed and non pre-exposed groups. However, the test concentrations during subsequent exposure did not show any significant difference for effect of thiacloprid on movement of *F. candida* in the groups that were not pre-exposed. Records of immobilized individuals indicated that thiacloprid also has significant effect on movement of springtails. With pre-exposure significant variation on proportion of stunned individuals was observed among test concentrations. The lowest effect was observed at 5 mg/kg (Fig 3.8) while the highest effect was observed in the control and 125 mg/kg during the subsequent exposure. However, there was no significant variation for this parameter among all doses of thiacloprid within the two groups. The highest difference (at $P<0.08$) in percent of stunned individuals was observed between 5 mg/kg without pre-exposure (lowest stunned data) and 0 mg/kg with pre-exposure (the highest stunned data). Movement showed different response pattern to thiacloprid with and without pre-exposure (Fig 3.9).

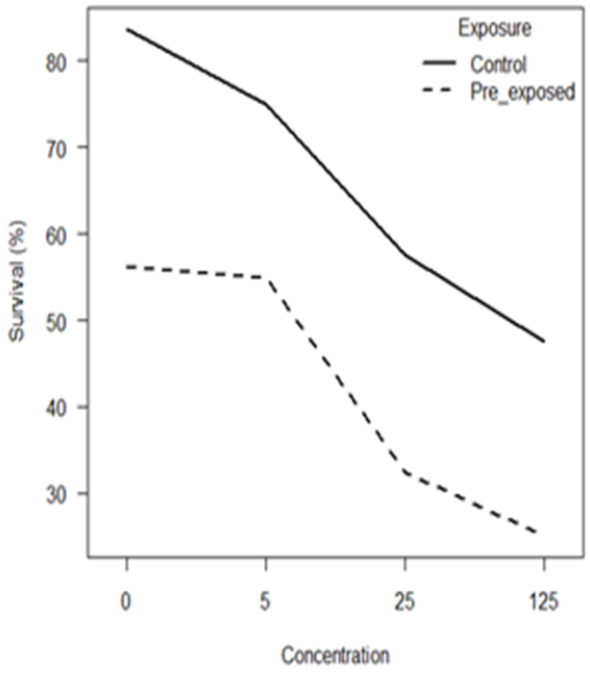

Fig 3.7: Response trend in survival (%) of *F. candida* over different concentrations (mg/kg) of thiacloprid with and without pre-exposure

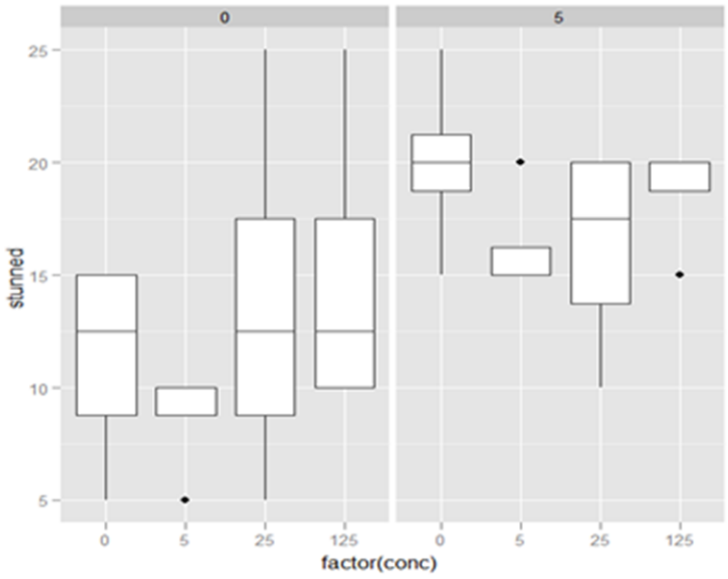

Fig 3.8: Percent of immobilized (stunned (%)) individuals in response to different concentrations (mg/kg) of thiacloprid, indicated as factor(conc).

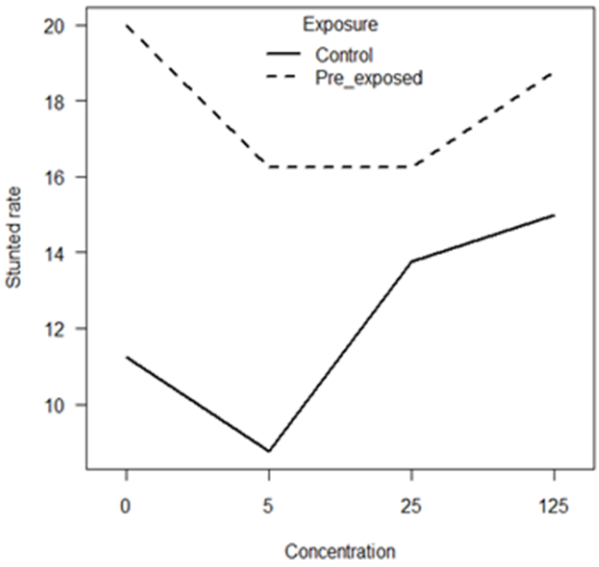

Fig 3.9: Response trend in mobility of *F. candida* over different concentrations (mg/kg) of thiacloprid with and without pre-exposure

Effect of pre-exposure on reproduction rate

Reproduction showed highly significant variation to pre-exposure ($F=43.23$; $P<0.01$), test concentrations ($F=10.34$; $P<0.01$) and their interaction ($F=4.91$; $P<0.01$). Effects of pre-exposure on reproduction occurred at all test concentration levels (Fig 3.10). The highest mean reproduction rate (11.07) was recorded from thiacloprid-free replicates of the control groups. There was continuous decreasing trend in reproduction for increasing doses of thiacloprid. Reproduction ability sharply falls with increasing concentration gradient starting from 5 mg/kg. Moreover, reproduction is highly affected by pre-exposure. Pre-exposure significantly reduced juvenile production in controls to 2.66. There was no significant difference on reproduction between the standard control and those exposed to 5 mg/kg among non-pre-exposed groups. However, pre-exposure significantly reduced juvenile production in controls to 2.66. However, the functional relationship of pre-exposed and non-exposed groups for reproduction rate narrows down to merge towards the higher doses (Fig 3.11).

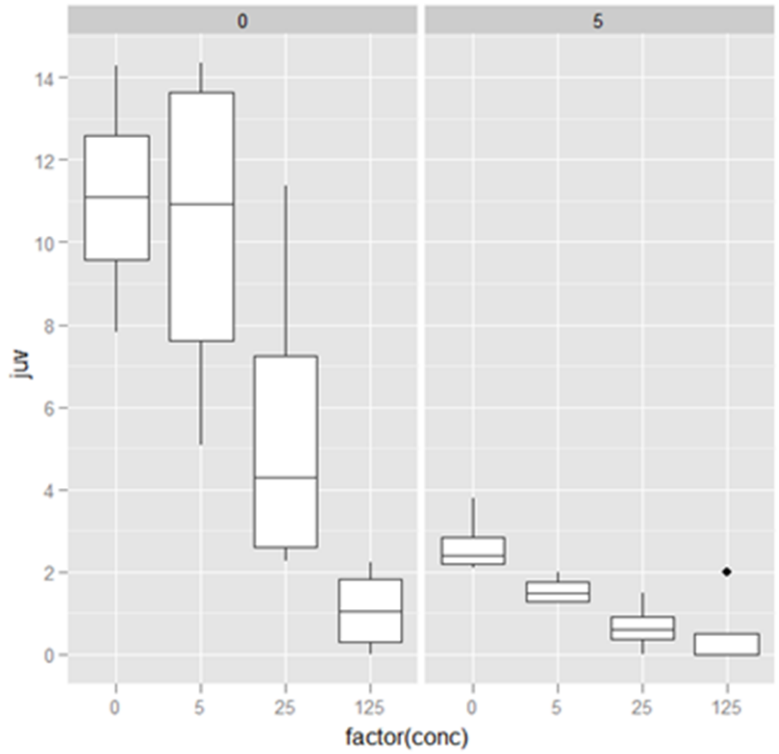

Fig 3.10: Effect of thiacloprid (mg/kg) on mean juvenile production per single *F. candida*

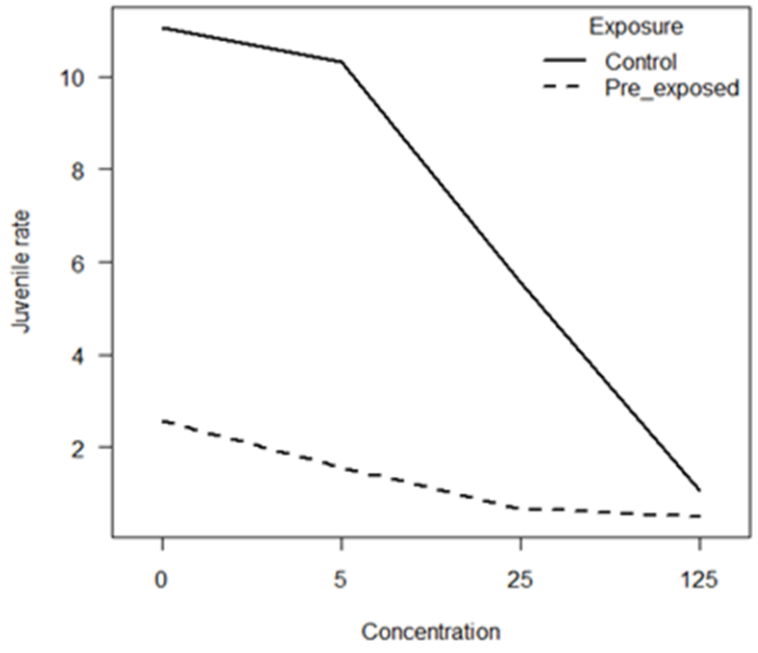

Fig 3.11: Response pattern in juvenile production by *F. candida* over different concentrations (mg/kg) of thiacloprid

Cross tolerance between cold exposure and thiacloprid

Experiment 1: Simultaneous exposure of thiacloprid and cold stress

There was 100 % mortality at 0 ^{0}C in all the concentrations of thiacloprid. Therefore, all replicates kept at 0 ^{0}C were discarded from the analysis. Hence, the results consider toxic effect of thiacloprid only at 7 ^{0}C and 15 ^{0}C.

Effects on survival and mobility

Comparative survival of springtails at 7 ^{0}C and 15 ^{0}C after exposure to the different test concentrations of thiacloprid is presented in Fig (3.12). ANOVA showed that both temperature (F=27.32; P<0.001) and concentration of thiacloprid (F=40.98; P<0.001) showed highly significant effects on survival of *F. candida* after exposure, with a higher overall mean survival recorded at 7 ^{0}C. Survival of individuals exposed to 25 mg/kg was as high as in the pesticide-free control at 7 ^{0}C while at the optimum temperature (15 ^{0}C), more than 50 % mortality was observed at this test concentration. Significant differences in survival were observed among the three doses of thiacloprid at 15 ^{0}C while at 7 ^{0}C only the 125 mg/kg concentration level showed lower survival than the control. However, the survival at 125 mg/kg and 7 ^{0}C was still higher than survival at 25 mg/kg and 15 ^{0}C (P<0.1).

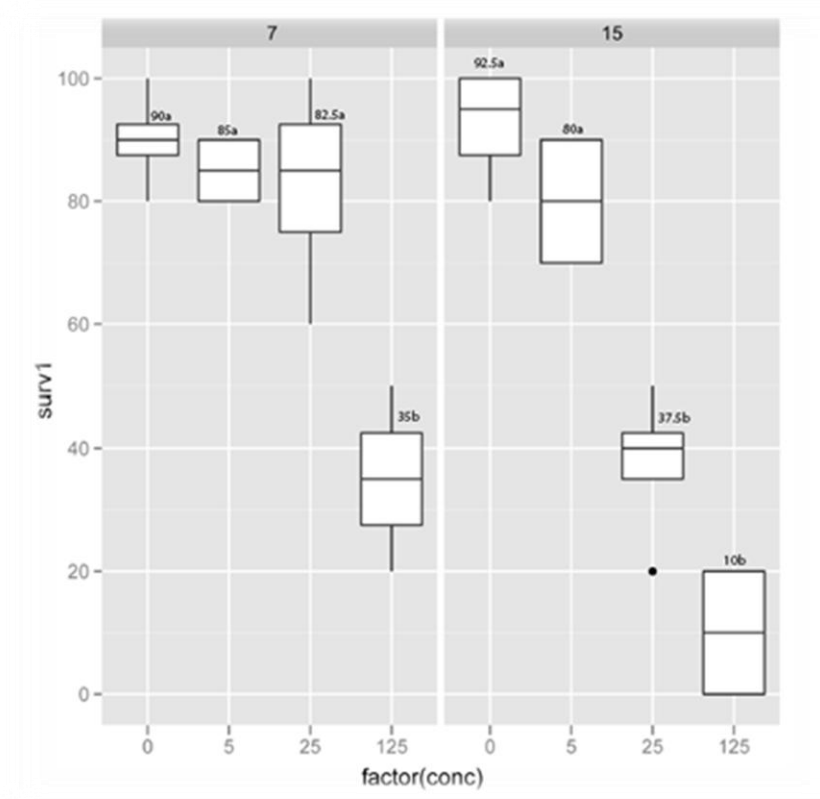

Fig 3.12: Combined effects simultaneous exposure to thiacloprid and soil temperature (^{0}C) on survival of *F. candida* two weeks after exposure

Keys:

factor (conc) = thiacloprid concentration (mg/kg)

surv1 = survival (%) of *F. candida* immediately after end of exposure period

7 and 15 stand for the two temperatures (^{0}C) at which evaluation of thiacloprid toxicity was made

Moreover, comparison of survival recorded immediately after the exposure period and one week after recovery showed significant delayed effects of temperature ($F=5.1491$; $P<0.05$) and its interaction with doses of thiacloprid ($F=4.3311$; $P<0.05$). This is depicted from comparative evaluation of survival measured immediately after end of the exposure (Fig 3.12) and survival recorded one week after recovery (Fig 3.13). Plots of the delayed effect which showed percent of late mortality at two temperatures (3.14) also support the argument here. Even though significant variation was not observed among test concentrations, there was a trend of increasing delayed effects in response to the first two doses (5 mg/kg and 25 mg/kg) at higher temperature. One week after recovery from exposure to the combined stress factors, survival of those groups exposed to 5 mg/kg at 15 ^{0}C, which did not have significant effect when evaluated immediately at the end of the exposure period was significantly reduced. Survival was significantly higher ($P<0.05$) at 25 mg/kg than 125 mg/kg immediately after the end of the exposure period at 15 ^{0}C (Fig 3.12). One week later the delayed effect decreased survival at 25 mg/kg to become statistically the same as that obtained at 125 mg/kg (Fig 3.13). However, any such delayed effect of the pesticide was not observed at 7 ^{0}C except for the dose 125 mg/kg.

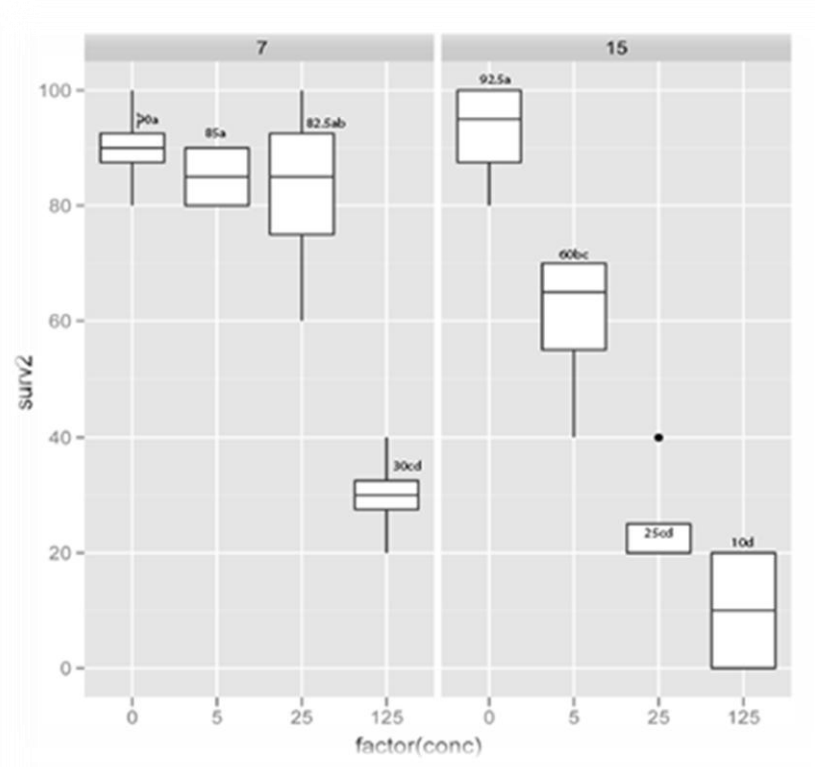

Fig 3.13: Combined effects of simultaneous exposure to thiacloprid and soil temperature one week after recovery from exposure

Keys:

factor(conc) = thiacloprid (mg/kg), Surv2 = survival one week after recovery.

7 and 15 are cold exposure temperatures (^{0}C).

The keys hold the same for all graphs of cross tolerance experiments

Low temperature also showed highly significant interaction with thiacloprid for all parameters F=5.958; P<0.001). The effect of this interaction is expressed in higher survival at 25 mg/kg of thiacloprid which was significantly at par with the control at 7 ^{0}C (Fig 3.12). This dose caused more than 60 % mortality at 15 ^{0}C.

Effects on reproduction

ANOVA for thiacloprid, temperature and their interaction also showed no significant difference in reproduction (egg laying). However, comparison between groups showed that those springtails exposed to thiacloprid at 7 ^{0}C gave numerically higher mean number of eggs per adult, observed one week after recovery from stress factors at 15 ^{0}C (Fig 3.15).

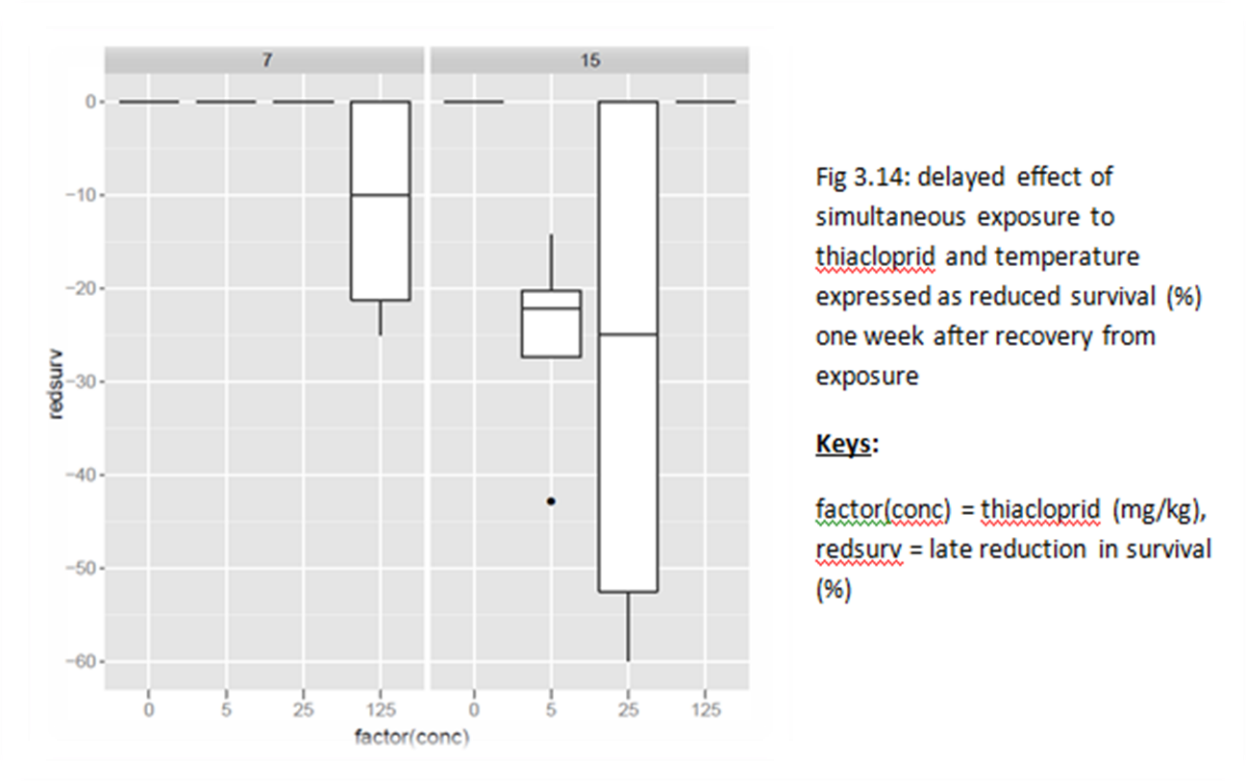

Fig 3.14: delayed effect of simultaneous exposure to thiacloprid and temperature expressed as reduced survival (%) one week after recovery from exposure

Keys:

factor(conc) = thiacloprid (mg/kg), redsurv = late reduction in survival (%)

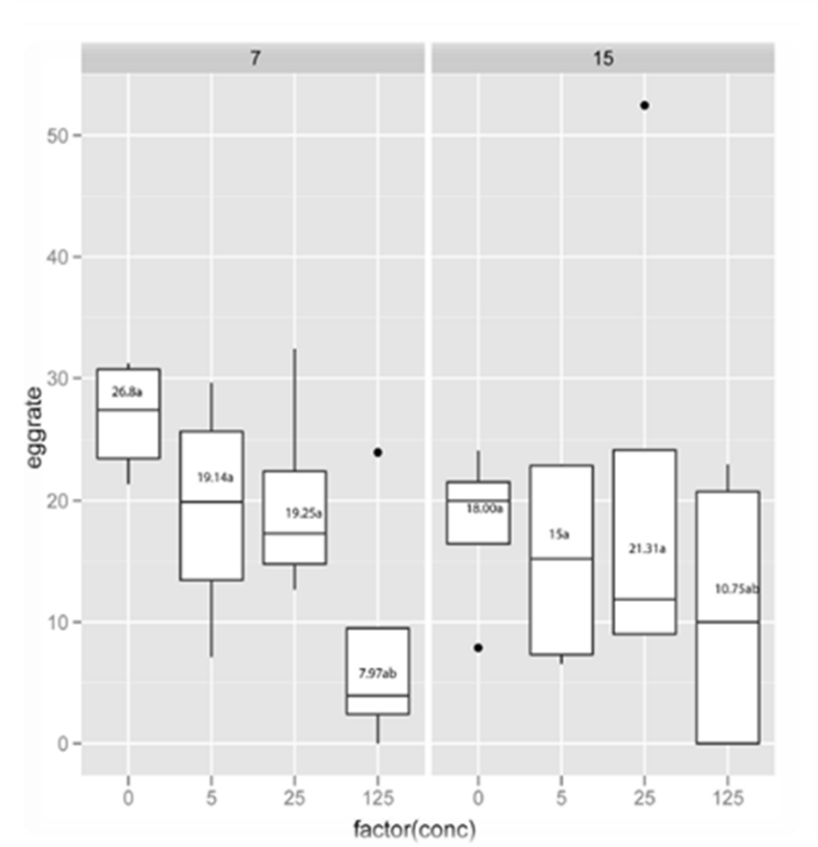

Fig 3.15: Combined effect of simultaneous exposure to thiacloprid and temperature on mean egg production capacity of *F. candida* measured one week after recovery from exposure

Keys: factor(conc) = thiacloprid (mg/kg); eggrate = mean number of eggs produced per single *F. candida*

those springtails exposed to thiacloprid at 7 ^{0}C gave numerically higher mean number of eggs per adult, observed one week after recovery from stress factors at 15 ^{0}C (Fig 3.15).

Experiment 2: Thiacloprid exposure prior to cold

Effects on survival and mobility

The results indicate that thiacloprid exposure did not have significant effect on subsequent cold tolerance ability of *F. candida*. All the treatments of thiacloprid including the thiacloprid-free control showed 100 % mortality at 0 ^{0}C. However, cold stress at 7 ^{0}C did not have significant effect on survival of *F. candida*. This has been also made evident from survival of thiacloprid-free control of this experiment kept at 7 ^{0}C and 15 ^{0}C, which did not show significant difference. Highly significant differences ($F=130.7778$; $P<0.001$) in survival was observed among all test concentrations at the two subsequent temperature exposures, and patterns of variation was the same for the two temperature exposures (Fig 3.16). The highest immediate effect of the pesticide was observed at 125 mg/kg. Under both conditions of temperature, mean mortality of 50 % was recorded near the dose 25 mg/kg.

Significant effect of temperature on survival was observed in the form of interaction with the pesticide ($F=3.5859$; $P<0.05$). This is depicted by a delayed effect of the pesticide following a subsequent exposure to low temperature. Delayed toxicity significantly varied for concentrations of thiacloprid ($F=5.7677$; $P<0.005$). Despite its effect on survival, there was no significant effect of the temperature-dose interaction for delayed toxicity. The highest numerical percent of late mortality (mortality recorded one week after recovery) was observed from the 25 mg/kg treatment level (22.5 %) at 15 ^{0}C (Fig 3.17). The significance of delayed effect can be inferred indirectly from group comparison of survival data before after exposure to treatments of temperature obtained after running Tukey mean separation. Tukey mean comparison between 25 mg/kg and 125 mg/kg at 15 ^{0}C gave significantly higher survival ($P<0.05$) for 25 mg/kg immediately after the end of exposure to thiacloprid, but before exposure to low temperature. However, Tukey means for these two doses were not significant when measured after exposure to cold stress. The highest delayed effect observed for 25 mg/kg was the cause for this reduced late survival at 15 ^{0}C. The mean survival of the species at this dose declined by 30 %.

Effects on reproduction

Thiacloprid and temperature affected reproduction both independently and combined. Figures 3.18 and 3.19 present the mean proportion of eggs laid and juveniles produced, respectively, from the springtails after they were exposed to cold stress following thiacloprid exposure. Highly significant differences in the number of eggs laid per individual adult were observed for temperature (70.321; $P<0.001$) and thiacloprid ($F=49.826$; $P<0.001$). Patterns of response in survival were also similar for concentrations of thiacloprid before after exposure to cold stress (Fig 3.16 and Fig 3.17). The mean number of juveniles hatched per individual adult also depended on thiacloprid exposure ($F=50.953$; $P<0.001$) and temperature ($F=100.491$; $P<0.001$). There was a highly significant interaction effect of the two stress factors with respect to both egg laying ($F=28.617$; $P<0.001$) and juvenile production ($F=50.956$; $P<0.001$). Interaction of temperature and thiacloprid resulted in higher variation in egg laying between thiacloprid-free replicates and those that received 5 mg/kg thiacloprid at 15 ^{0}C. These treatments did not give significant difference at 7 ^{0}C.

Even though *F. candida* were able to lay eggs whether kept thiacloprid-free or treated with 5 mg/kg, they were not able to produce significant difference in number of juveniles compared to the higher concentrations. At 15 ^{0}C, the difference in number of juveniles per *F. candida* recorded from replicates of 5 mg/kg and the control was highly significant ($P<0.0001$).

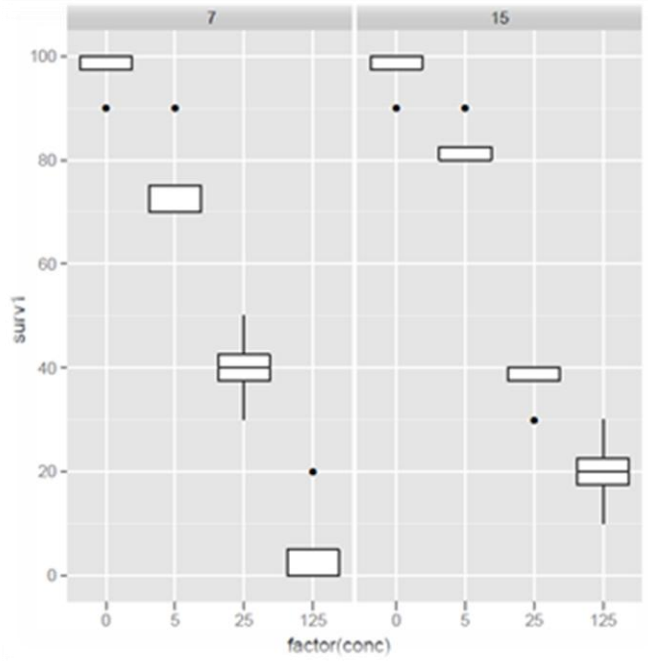

Fig3.16: Survival (%) of *F. candida* after exposure to thiacloprid for two weeks but prior to cold exposure

Keys: factor(conc) = thiacloprid (mg/kg); surv1 = survival (%) after exposure to thiacloprid, but prior to subsequent exposure to cold stress

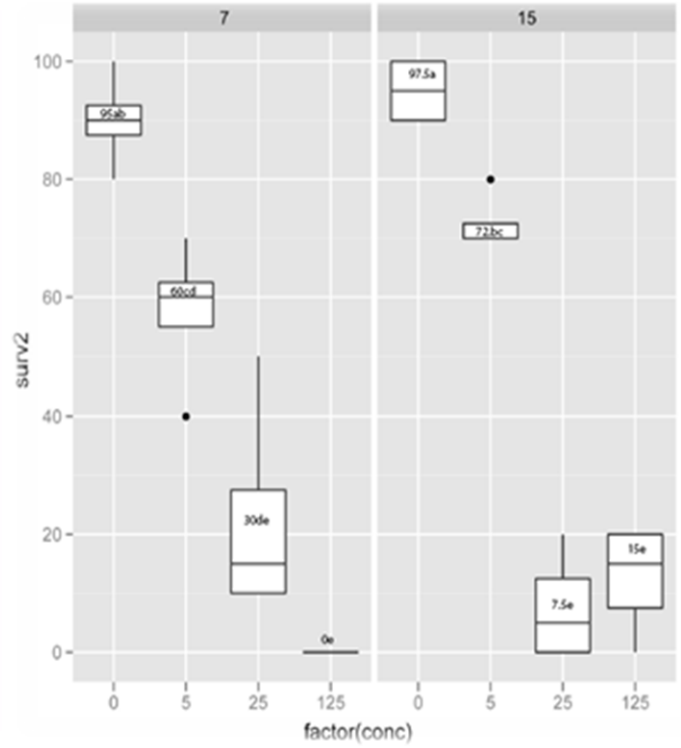

Fig 3.17: Effect of thiacloprid followed by exposure cold stress on survival of *F. candida*

Keys: factor(conc) = thiacloprid (mg/kg); surv2 = survival (%) of *F. candida* after exposure to thiacloprid followed by two weeks cold stress (each exposure periods for two weeks)

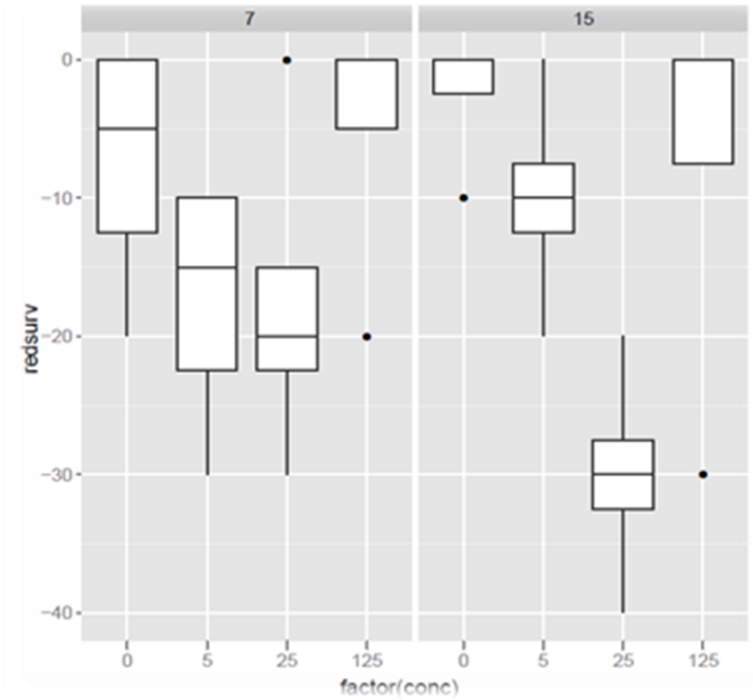

Fig 3.18: delayed effects of prior exposure to thiacloprid on survival of *F. candida* at two temperatures

Keys: factor(conc) = thiacloprid (mg/kg); redsurv = reduction in survival (%) one week after recovery from the subsequent exposure to thiacloprid and cold stress, respectively.

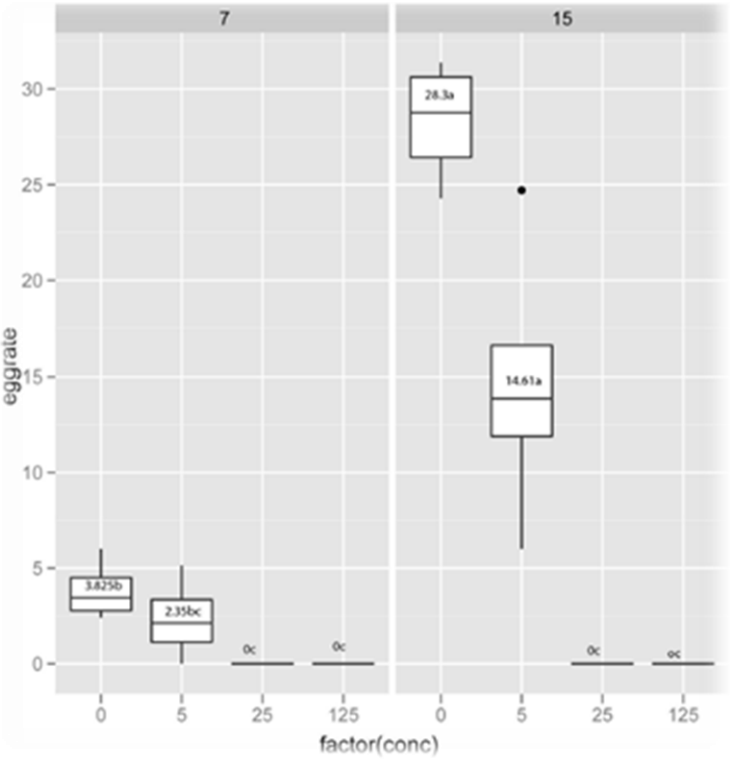

Fig 3.19: Mean number of eggs per single *F. candida* recorded in two weeks at two temperatures following two weeks prior exposure to thiacloprid

Keys: factor(conc) = thiacloprid (mg/kg); eggrate = mean number of eggs produced per single *F. candida*

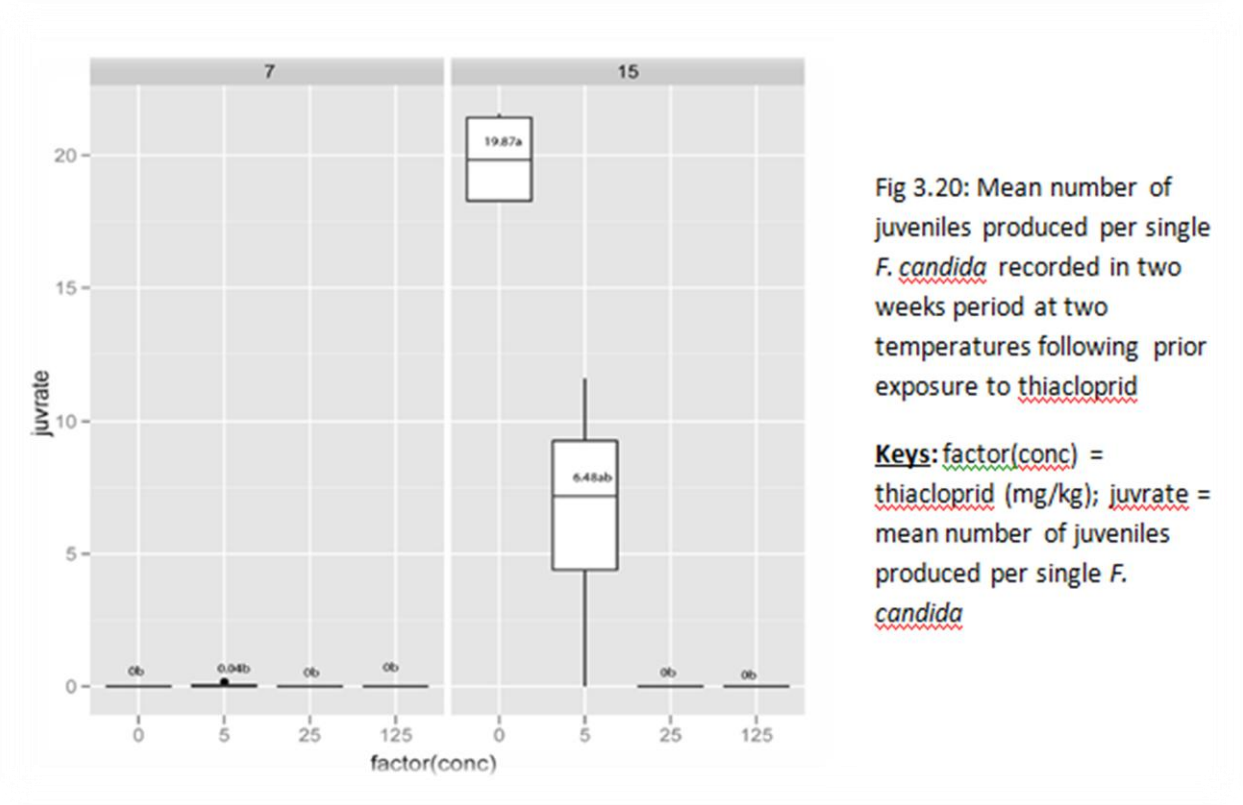

Fig 3.20: Mean number of juveniles produced per single *F. candida* recorded in two weeks period at two temperatures following prior exposure to thiacloprid

Keys: factor(conc) = thiacloprid (mg/kg); juvrate = mean number of juveniles produced per single *F. candida*

Experiment 3: Cold exposure prior to thiacloprid

Like in the previous two experiments, 100 % mortality was recorded from all treatments of thiacloprid kept at 0 ^{0}C. Thus, results from 0 ^{0}C were discarded from the analysis. Cold exposure at 7 ^{0}C did not have significant effect on survival of the springtails when applied prior to thiacloprid. All replicates showed 100 % survival at both temperature conditions.

Significant ANOVA was observed only for doses of thiacloprid on survival immediately after end of exposure ($F=140.4133$; $P<0.0001$ and Fig 3.21) and one week after end of exposure ($F=284.0909$; $P<0.0001$ and Fig 3.22). Responses adopt the same trend for the different doses of thiacloprid at the two temperature conditions (Fig 3.21). Tukey multiple comparisons of means showed significant variation among all test concentrations in both temperatures except 5 mg/kg and the control. Survival significantly declined with increasing dose beyond 5 mg/kg. Moreover, comparison of survival for any given dose between the two temperatures did not give significant difference.

As indicated in Fig 3.22, thiacloprid caused continued decline in survival after the species were allowed to recover for one week from the pesticide and cold exposure. However, the proportion of decrease in survival was closely similar. This was also indicated by figure 3.23 for delayed effect on survival. ANOVA for delayed effect on survival showed no

significant variation among doses. No significant interaction between temperature and doses of thiacloprid was also observed for all parameters measured.

Results for reproduction bioassays are indicated in Fig 3.24. Significant effect on egg laying was observed only for doses of thiacloprid (ANOVA, F =47.1859; P<0.0001). No egg laying was recorded from 125 mg/kg. Temperature did not show any effect on egg laying capacity of *F. candida*. Proportion of eggs laid at the two different temperatures was statistically equal for any given dose of thiacloprid within the test concentrations. ANOVA also showed no statistical difference in number of eggs laid at 0 mg/kg and 5 mg/kg.

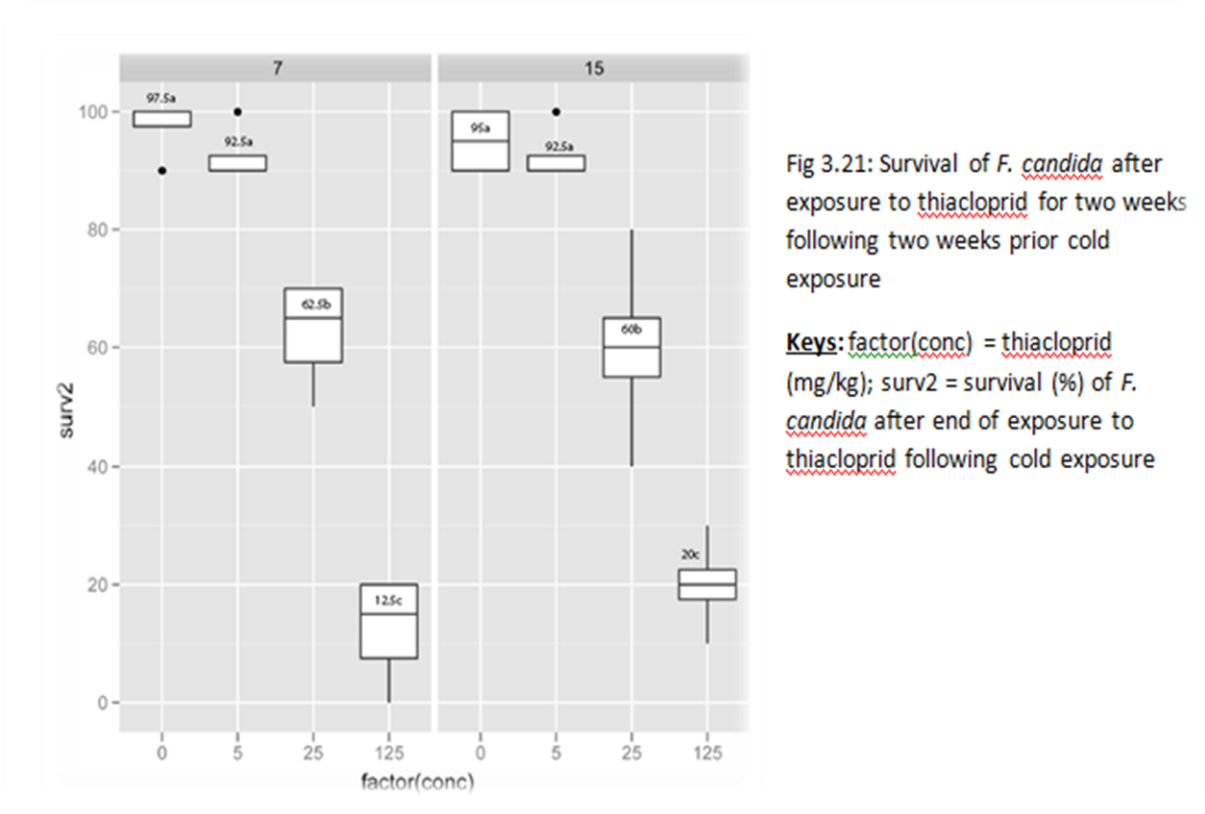

Fig 3.21: Survival of *F. candida* after exposure to thiacloprid for two weeks following two weeks prior cold exposure

Keys: factor(conc) = thiacloprid (mg/kg); surv2 = survival (%) of *F. candida* after end of exposure to thiacloprid following cold exposure

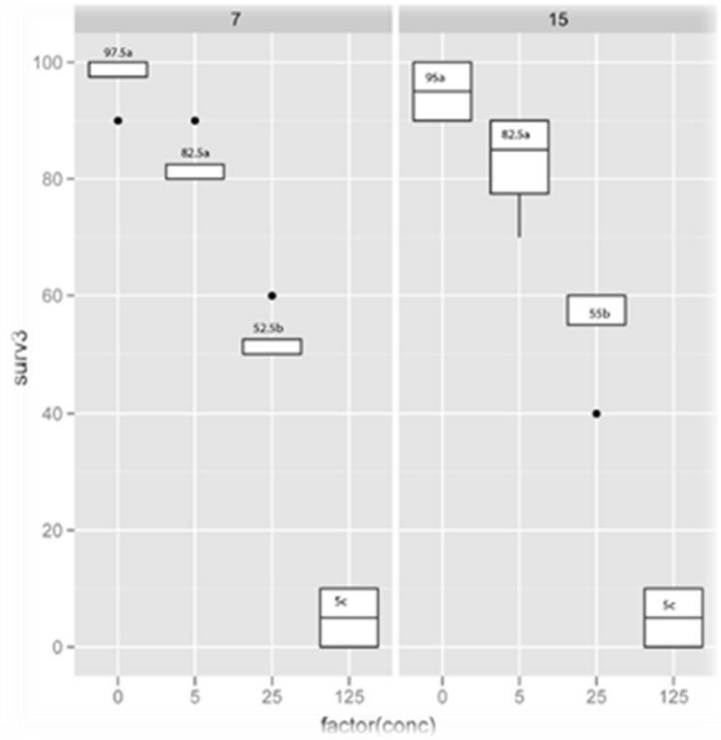

Fig 3.22: Survival of *F. candida* one week after recovery from exposure to thiacloprid for two weeks following two weeks prior cold exposure

Keys: factor(conc) = thiacloprid (mg/kg); surv3 = survival (%) of *F. candida* after recovery from the successive cold stress and thiacloprid exposure

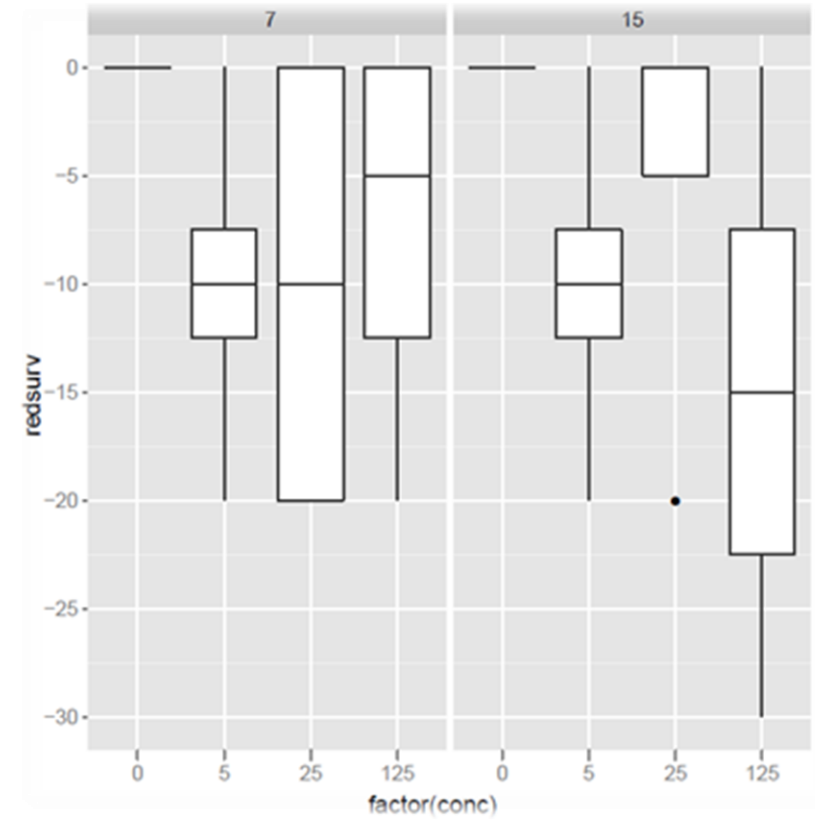

Fig 3.23: delayed effect of thiacloprid following two cold exposure observed as reduction in one week after recovery

Keys: factor(conc) = thiacloprid (mg/kg); redsurv = reduction in survival (%) one week after recovery from the subsequent exposure to cold and thiacloprid.

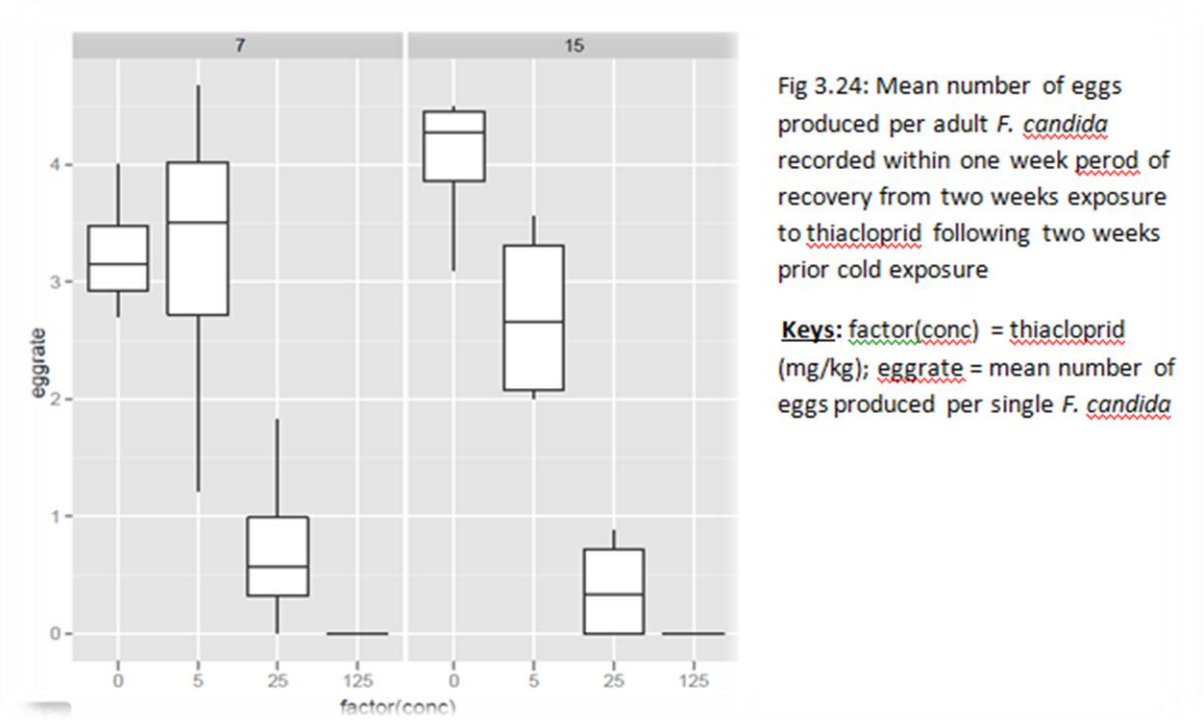

Fig 3.24: Mean number of eggs produced per adult *F. candida* recorded within one week perod of recovery from two weeks exposure to thiacloprid following two weeks prior cold exposure

Keys: factor(conc) = thiacloprid (mg/kg); eggrate = mean number of eggs produced per single *F. candida*

Discussion

Pre-exposure to lower concentration of thiacloprid reduced the tolerance of *F. candida* to subsequent exposure to higher concentrations of the compound. According to the dose-response relationship, pre-exposure reduced the LC50 by about 61 times to the dose without pre-exposure. As indicated from the results, pre-exposure may cause this through various mechanisms. Martikainen and Rantalainen (1999) suggested that reduced movement ability caused by dimethoate could have beneficiary effects to *F. candida* as it enabled the species to reallocate resource for body growth. However, the findings of this study suggest the opposite as a positive correlation between reduced movement and mortality was observed. An effect of reduced movement may depend on type of contaminant and the mechanism causing it; some contaminants may cause paralysis indicated by uncoordinated movement of appendages. Many of the immobilized *F. candida* recorded in this study showed signs of paralysis and uncoordinated movement of appendages.

Moving away from contaminated spots can be the mechanism by which springtails survive contaminated environments. However, reduced ability of active movement will not enable the species to escape the contaminated habitats. Hence, they may become subject to contaminated environments for a prolonged period of time. Under such circumstances, even lower doses may be enough to cause higher mortality and highly reduced fitness in general. This may be more severe with persistence of the compound and other environmental factors that facilitate bioavailability and uptake. Thiacloprid can persist in

the environment up to two months (USEPA, 2003), which is relevant to the time frame used for the experiments reported here.

The findings of this study did not comply with the initial hypothesis of the study which assumed no change in tolerance following pre-exposure. It is also contrast to the findings of Valandoost (1999) who reported development of enhanced tolerance to permethrin by mosquitos following pre-exposure to a sub-lethal dose of the compound. Tolerance usually involves physiological mechanisms that may be controlled by genetic systems which can in turn be triggered by exposure to contaminants. The possible physiological changes in response to an environmental stressor may induce tolerance or susceptibility to another stress factor. The net effect of such combined stress factors may depend on the physiological system investigated, of the organism, and possibly also on the environmental behavior of the contaminant.

Both the survival and reproduction bioassays support the theory that says duration of exposure has significant impact on the toxicity of chemicals (van Leeuwen in van Leeuwen and Vermeire, 2007: 4-5; Wright and Welbourn, 2008; Valandoost, 1999). The subsequent test exposed the springtails to thiacloprid at three different doses for two weeks. In the case that involves pre-exposure, the total duration of thiacloprid exposure was four weeks (two weeks for pre-exposure followed by two weeks of exposure to additional doses). The significant reduction in survival and reproduction of springtails that were pre-exposed indicates that there is a prolonged effect of the pre-exposure dose. Survival was equally affected at 5 mg/kg with pre-exposure compared to 25 mg/kg without pre-exposure. This implies presence of additive or cumulative effect of pre-exposure on *F. candida*. The findings thus conclude that *F. candida* cannot be pre-acclimated to thiacloprid, at least not with the concentration tested here.

The recorded effects on movement and reproduction ability indicate that thiacloprid has the ability to cause chronic toxicity to *F. candida*. Usually chronic toxicity develops over longer and more frequent exposures to sub lethal doses. When the reproduction capacity of individuals from the pre-exposed group that were kept thiacloprid free control during subsequent exposure was compared with those replicates from no previous exposure but subsequently exposed to 5 mg/kg, marked reduction in reproduction potential and movement ability was obtained from the first group. In the first group the total time before measurement of data since pesticide exposure was four weeks (since time of exposure to pre-exposure concentration) while the second group were exposed only for two weeks. This explains significance of duration of exposure period in chronic toxicity.

Various authors mentioned the importance of considering a combination of as many stress factors in the environment as possible in order to give environmental risk assessment studies a broader ecological relevance (Sjursen and Holmstrup, 2004; Holmstrup et al.,

2008; Bednarska, et al., 2009, Holmstrup et al., 2010). In many instances, climatic stress factors are suspected to have synergetic interactions with toxic stress (Sjursen et al., 2001). Synergetic effects of cold stress have been reported with some chemicals, especially metals (Holmstrup et al., 2008; Slotsbo et al., 2009). However, combined effect of climatic stress and toxic agents may vary depending on how they are combined. In a study that considered combined effect of exposure to sub lethal temperature and sub lethal mercury, higher mortality was recorded when the exposure was made simultaneously than when applied one after the other (Slotsbo et al., 2009). In another study, Sjursen and Holmstrup (2004) found different results on the combined effect of drought and pyrene when the two stress factors are combined in different order. The authors found synergetic effect when pyrene was applied prior to drought, but not the reverse. Reflecting the findings above, the study design of the experiments reported here aimed to test for cross tolerance with low temperature and thiacloprid combined in three different orders (simultaneous exposures, thiacloprid prior to temperature and temperature prior to thiacloprid). However, as none of the experiments proved presence of synergetic effect between the two stress factors, the he results imply that neither thiacloprid nor low temperature induce tolerance against one another in subsequent exposure set-ups. The results do however indicate that low temperature interfered with the toxicity of thiacloprid when they are applied simultaneously.

Moderately low temperatures did not show any significant effect on survival while 100 % mortality was recorded at 0 ^{0}C. When thiacloprid was applied first, subsequent exposure to low temperature did not prevent the damaging effect of the toxicant that was already introduced into the body. In this case the pesticide was already absorbed into the body of the springtails while they were kept at 15 ^{0}C during the period when they were exposed to thiacloprid. The most distinguished significance of temperature was observed in the experiment with a simultaneous exposure to cold and thiacloprid, where low temperature reduced the toxicity by about a factor of seven. The LC50 values at 15 ^{0}C and 7 ^{0}C determined by the dose-response relationship were 9.81 and 72.24, respectively (Table 3.1). This implies that low temperature of 7 ^{0}C increases the LC50 by seven folds of the LC50 at 15 ^{0}C. However, according to results of ANOVA for survival data at the two temperatures (Fig 3.12 and Fig 3.13), the dose that gave about 50 % mortality at 7 ^{0}C was 5 times higher than the dose required at 15 ^{0}C. The results in general are contradictory from the earlier reports by various authors who reported increased toxicity of different chemicals when combined with cold stress (Holmstrup et al., 2008). Low temperature in our case instead reduced the toxicity of thiacloprid when applied simultaneously.

Significant interaction between temperature and thiacloprid resulted in more continued mortality at 15 ^{0}C for the experiment with thiacloprid applied prior to low temperature. The springtails set for subsequent exposure to low temperature absorbed the pesticide in

the two week time of thiacloprid exposure when they were kept at 15 ^{0}C. Any mortality recorded after they were extracted from pesticide exposure and transferred to temperature treatment was expected to reflect a delayed effect of the pesticide. Delayed effects were actually observed for the test concentrations of 5 mg/kg and 25 mg/kg both at 7 ^{0}C and 15 ^{0}C. Even though results are non-significant, lower temperature exhibited tendency of reducing the delayed effect. This may imply that at 7 ^{0}C, the springtails may slow down their metabolic rate that affected also toxic development of thiacloprid. On the other hand no delayed effect was observed for the first two doses. Prior cold exposure also did not show any difference in delayed effect of the pesticide.

In some cases, exposure to some climatic stress factors prior to chemical stress was found to provide tolerance to the organism to the chemical substances. As reported by Sjursen and Holmstrup (2004) this may be associated with induction of internal compounds that can combat the toxic development of the compound in the body. Interactive effect of temperature on toxicity of chemical pollutants may vary depending on the chemical nature of the pollutant and physiological system of the organism at different temperature (Martikainen and Rantalainen, 1999). Temperature is the most important climatic factor that continuously varies, especially in temperate climates and affects physiology of organism. However, findings of this study cannot be related to any such assumptions since our results of the cross tolerance did not include the analysis from cooling temperatures for *F. candida*. It is difficult to infer any assumptions about the interaction of the pesticide with cold stress at 0 ^{0}C since all the springtails that were maintained at 0 ^{0}C were dead and thus discarded from the analysis. The method of burying the springtails under crystals of ice may be the source of technical error for such absolute mortality of the species at this temperature which is reported by others to be within the range of tolerance of the species. The ice was found melting in about two and three days. This may result in change in temperature. In such conditions of temperature frequent flux, the species may not have time to adapt to the freezing temperature.

Hindering bioavailability (Sjursen and Holmstrup, 2004; Bindesbøl, 2008) and denying uptake (Sjursen and Holmstrup, 2004) are the main general mechanisms how temperature interferes with toxicity of chemical pollutants in the environment prior to their entry into the body. However, inside the body temperature affects toxicity of contaminants through altering metabolism of the organism which may involve genetic systems (Martikainen and Rantalainen, 1999; Sjursen and Holmstrup, 2004). There may also be cases where low temperature facilitates entry of toxic compounds to target cellular contents by destroying cellular structures such as membranes due to ice formation in the cell (Bahrndorff et al., 2009). However, this is possible at freezing temperatures. Thus, this assumption does not apply for our case where applied treatments did not include freezing. Moreover, springtails naturally avoid ice formation due to cold by virtue of their freeze avoiding mechanism

(Bahrndorff et al., 2009). Taking these concepts into consideration and comparing the results of simultaneous exposure with the other two where thiacloprid and low temperature were imposed one after the other, it is possible to conclude that low temperature may impede toxicity of thiacloprid before it's absorption in the body. Reduced metabolism may also hinder or retard toxic transformation of thiacloprid even after absorption.

To estimate whether this reduced uptake is due reduced metabolism of the organism or low bioavailability of the pesticide, it is worth to compare activity of the organism and delayed effect of the pesticide under the two temperatures. Cold exposure at 7 ^{0}C did not affect activity of the species. This has been consistently confirmed by similar survival values of control individuals of the three experiments at 7 ^{0}C and 15 ^{0}C. Tests where exposure to temperature was applied prior to thiacloprid also gave 100 % survival at the two temperatures. Moreover, delayed effects observed at these two temperature levels imply that the springtails were able to maintain their active metabolism at 7 ^{0}C and 15 ^{0}C. Hence, the effects of temperature are most probably exerted on thiacloprid reducing its bioavailability. These finding is in agreement with Sjursen and Holmstrup (2004) who found reduced toxicity of pyrene at low temperature due to low bioavailability and hence reduced uptake. Even though the results in this study are non-significant, declining trend in survival across increasing doses of thiacloprid during simultaneous exposure also supported this assumption. Slightly higher mortality was recorded with increasing doses. This can be related to increasing bioavailability of the pollutant in the soil with increasing doses.

Naturally low temperature delays fecundity of *F. candida*. Highly significant interaction of low temperature with thiacloprid was observed for reproduction traits. The impact of the interaction appears to be higher on reproduction than on survival. Interactive effect of low temperature was observed on hatching, but not on egg laying. This resulted in no hatching at 7 ^{0}C within the two weeks period of data collection. At 15 ^{0}C, the number of juveniles produced by springtails that had been exposed to 5 mg/kg was significantly lower than in the controls at 0 mg/kg at the same temperature. This dose of thiacloprid at 15 ^{0}C did not have significant difference in egg laying. These results may suggest that the interactive effect of temperature is expressed on hatching.

Thiacloprid is reported to exhibit both acute and chronic toxicity (USEPA, 2003). However, our experiments may not conclude about the chronic toxicity of the pesticide as they included short periods for evaluation of reproduction on various parameters such as time of egg laying, incubation period and fecundity. Therefore, it is important that further studies consider evaluation of data for chronic toxicity.

The LC50 values determined from the dose-response function for the different experiments did not show consistency. Consistent results were found for the cross tolerance experiments. Higher LC50 values in the range finding test may be due to shorter period of exposure of the springtails. However, extremely higher LC50 values in the pre-exposure experiment may involve technical error. Hence this needs to be confirmed further.

Practical implications for ecological risk assessment and use of thiacloprid in pest management

Generally, the results of these findings have practical implications with regard to pest management actions which have to take various aspects of the environment into consideration. Pre-exposure experiments prove that even though initial exposure to sub lethal doses did not show immediate significant effect, it showed significant cumulative effect during subsequent exposure to the same sub lethal dose. In all cases of cross tolerance experiments, thiacloprid at 5 mg/kg was able to reduce survival due to its delayed effect. The implication here is that subsequent exposures to the same sub-lethal dose reduce survival in additive manner. According to the calculated LC50 values and ANOVA for survival data the dose that killed 50 % of the population is significantly reduced with pre-exposure. Under such circumstances splitting pesticide application with lower doses may be used to reduce environmental risk. Moreover, use of split applications with lower doses may have economic significance in terms of reducing the cost of the pesticide itself.

On the other hand, low temperature displayed significant increase in the LC50 of thiacloprid. It required 125 mg/kg thiacloprid to cause 70 % average mortality of *F. candida* at 7 ^{0}C. This is the dose which gave 90 % mortality at 15 ^{0}C. This result implies consideration of application season of thiacloprid as a pesticide in environments where springtails are found. Considering physiology and thus relative response of organisms to a given pesticide at different temperatures (seasons) also provides an important guide to synchronize application season of the pesticide relative to temperature. In this case for example, thiacloprid may be applied during cold seasons that may not affect springtails but other target pests which may be susceptible to the pesticide at low doses under that temperature. However, this requires further investigation as to whether the reduced mortality at colder temperature is due to reduced bioavailability, reduced uptake, or both. The suggestion given would be more practical if the reduced toxicity is due to reduced uptake by the organism due to metabolic rate. Hence, further investigations to address this issue properly should include more physiological and molecular bioassays. Finally, it is important to consider different temperature conditions to provide more practical recommendations from ecological risk assessment studies.

APPENDIX – ANOVA tables for the different experiments

1. **Pre-acclimation**
   - **1.1. Survival**
     - **a. With interaction**

summary(x1.aov <- aov(survive ~ as.factor(pre) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(pre)	1	4512.5	4512.5	31.7363	8.445e-06 ***
as.factor(conc)	3	6184.4	2061.5	14.4982	1.349e-05 ***
as.factor(pre):as.factor(conc)	3	62.5	20.8	0.1465	0.9309
Residuals	24	3412.5	142.2		

     - **b. Without interaction, but combined effect**

summary(x1.aov <- aov(survive ~ as.factor(pre) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(pre)	1	4512.5	4512.5	35.061	2.604e-06 ***
as.factor(conc)	3	6184.4	2061.5	16.017	3.522e-06 ***
Residuals	27	3475.0	128.7		

   - **1.2. Stunned**
     - **a. With interaction**

summary(x1.aov <- aov(stunned ~ as.factor(pre) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(pre)	1	253.12	253.125	9.9184	0.004341 **
as.factor(conc)	3	81.25	27.083	1.0612	0.383968
as.factor(pre):as.factor(conc)	3	53.13	17.708	0.6939	0.564793
Residuals	24	612.50	25.521		

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b. Without interaction, but combined effect

summary(x1.aov <- aov(stunned ~ as.factor(pre) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(pre)	1	253.12	253.125	10.2676	0.003462 **
as.factor(conc)	3	81.25	27.083	1.0986	0.366763
Residuals	27	665.63	24.653		

1.3. Juvrate

a. With interaction

summary(x1.aov <- aov(juv ~ as.factor(pre) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(pre)	1	255.606	255.606	43.2311	8.434e-07 ***
as.factor(conc)	3	183.427	61.142	10.3411	0.0001476 ***
as.factor(pre):as.factor(conc)	3	87.093	29.031	4.9101	0.0084406 **
Residuals	24	141.901	5.913		

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b. Without interaction, but combined effect

summary(x1.aov <- aov(juv ~ as.factor(pre) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(pre)	1	255.61	255.606	30.1378	8.194e-06 ***
as.factor(conc)	3	183.43	61.142	7.2091	0.001051 **
Residuals	27	228.99	8.481		

2. Cross tolerance

2.1. Experiment 1: Simultaneous exposure to thiacloprid and cold stress

2.1.1. Survival before the springtails are kept to normal

a. With interaction

summary(x1.aov <- aov(surv1 ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	2628.1	2628.1	19.5581	0.0001804 ***
as.factor(conc)	3	22584.4	7528.1	56.0233	5.491e-11 ***
as.factor(temp):as.factor(conc)	3	2734.4	911.5	6.7829	0.0017971 **
Residuals	24	3225.0	134.4		

b. Combined effect, without intrxn

summary(x1.aov <- aov(surv1 ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	2628.1	2628.1	11.907	0.001854 **
as.factor(conc)	3	22584.4	7528.1	34.108	2.502e-09 ***
Residuals	27	5959.4	220.7		

2.1.2. Survival one week after the springtails are kept to normal

a. With interaction

summary(x1.aov <- aov(surv2 ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	5000	5000.0	40.6780	1.355e-06 ***
as.factor(conc)	3	22163	7387.5	60.1017	2.627e-11 ***
as.factor(temp):as.factor(conc)	3	3675	1225.0	9.9661	0.0001878 ***
Residuals	24	2950	122.9		

b. Combined effect, but no intrxn

summary(x1.aov <- aov(surv2 ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	5000	5000.0	20.377	0.0001124 ***
as.factor(conc)	3	22163	7387.5	30.108	9.2e-09 ***
Residuals	27	6625	245.4		

2.1.3. Delayed effect (late mortality)

a. With interaction

summary(x1.aov <- aov(surv1 ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	2628.1	2628.1	19.5581	0.0001804 ***
as.factor(conc)	3	22584.4	7528.1	56.0233	5.491e-11 ***
as.factor(temp):as.factor(conc)	3	2734.4	911.5	6.7829	0.0017971 **
Residuals	24	3225.0	134.4		

---

b. Combined effect, but no intrxn

summary(x1.aov <- aov(surv1 ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	2628.1	2628.1	11.907	0.001854 **
as.factor(conc)	3	22584.4	7528.1	34.108	2.502e-09 ***
Residuals	27	5959.4	220.7		

2.1.4. Eggrate

a. With interaction

summary(x1.aov <- aov(eggrate ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	38.81	38.808	0.2973	0.5906
as.factor(conc)	3	802.71	267.570	2.0499	0.1336
as.factor(temp):as.factor(conc)	3	171.05	57.017	0.4368	0.7287
Residuals	24	3132.66	130.527		

b. Combined, but no intrxn

summary(x1.aov <- aov(eggrate ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	38.8	38.808	0.3172	0.5780
as.factor(conc)	3	802.7	267.570	2.1868	0.1127
Residuals	27	3303.7	122.360		

2.2. ANOVA for experiment that involve exposure to thiacloprid prior to cold stress

2.2.1. Survival

a. With interaction

mary(x1.aov <- aov(surv2 ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	153	153.1	1.4848	0.23486
as.factor(conc)	3	40459	13486.5	130.7778	5.28e-15 ***
as.factor(temp):as.factor(conc)	3	1109	369.8	3.5859	0.02844 *
Residuals	24	2475	103.1		

b. Without interaction, but combined effect

> summary(x1.aov <- aov(surv2 ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	153	153.1	1.1534	0.2923
as.factor(conc)	3	40459	13486.5	101.5894	8.025e-15 ***
Residuals	27	3584	132.8		

2.2.2. Delayed effect (late mortality)

a. With interaction

> summary(x1.aov <- aov(delayed ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	3.12	3.12	0.0303	0.863264
as.factor(conc)	3	1784.38	594.79	5.7677	0.004061 **
as.factor(temp):as.factor(conc)	3	484.37	161.46	1.5657	0.223600
Residuals	24	2475.00	103.12		

---

b. Without interaction, but combined effect

summary(x1.aov <- aov(delayed ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	3.12	3.12	0.0285	0.867171
as.factor(conc)	3	1784.38	594.79	5.4266	0.004717 **
Residuals	27	2959.37	109.61		

2.2.3. Eggrate

a. With interaction

summary(x1.aov <- aov(eggrate ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	674.82	674.82	70.321	1.358e-08 ***
as.factor(conc)	3	1434.45	478.15	49.826	1.850e-10 ***
as.factor(temp):as.factor(conc)	3	823.85	274.62	28.617	4.259e-08 ***
Residuals	24	230.31	9.60		

---

b. Without interaction, but combined effect

> summary(x1.aov <- aov(eggrate ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	674.82	674.82	17.284	0.0002915 ***
as.factor(conc)	3	1434.45	478.15	12.247	3.049e-05 ***
Residuals	27	1054.16	39.04		

2.2.4. Juvrate

a. With interaction

summary(x1.aov <- aov(juvrate ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	346.17	346.17	100.491	4.686e-10 ***
as.factor(conc)	3	526.57	175.52	50.953	1.470e-10 ***
as.factor(temp):as.factor(conc)	3	526.61	175.54	50.956	1.469e-10 ***
Residuals	24	82.68	3.44		

---

b. Without interaction, but combined effect

summary(x1.aov <- aov(juvrate ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	346.17	346.17	15.3405	0.0005515 ***
as.factor(conc)	3	526.57	175.52	7.7782	0.0006716 ***
Residuals	27	609.28	22.57		

2.3. ANOVA for experiment that involve cold exposure prior to thiacloprid

2.3.1. Survival

a. With interaction

summary(x1.aov <- aov(surv2 ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	0	0.0	0.0000	1.0000
as.factor(conc)	3	39062	13020.8	284.0909	<2e-16 ***
as.factor(temp):as.factor(conc)	3	25	8.3	0.1818	0.9077
Residuals	24	1100	45.8		

b. Without interaction, but combined effect

summary(x1.aov <- aov(surv2 ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	0	0.0	0.0	1
as.factor(conc)	3	39062	13020.8	312.5	<2e-16 ***
Residuals	27	1125	41.7		

2.3.2. Delayed effect (late mortality)

a. With interaction

summary(x1.aov <- aov(delayed ~ as.factor(temp) * as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	3.13	3.125	0.04	0.84317
as.factor(conc)	3	609.37	203.125	2.60	0.07552 .
as.factor(temp):as.factor(conc)	3	159.38	53.125	0.68	0.57288
Residuals	24	1875.00	78.125		

b. Without interaction, but combined effect

summary(x1.aov <- aov(delayed ~ as.factor(temp) + as.factor(conc), data = A))

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	3.13	3.125	0.0415	0.84015
as.factor(conc)	3	609.37	203.125	2.6959	0.06578 .
Residuals	27	2034.38	75.347		

2.4. Eggrate

a. With interaction

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	0.003	0.0028	0.0052	0.9430
as.factor(conc)	3	76.338	25.4460	47.1859	3.23e-10 ***
as.factor(temp):as.factor(conc)	3	2.005	0.6682	1.2391	0.3174
Residuals	24	12.942	0.5393		

b. Without interaction, but combined effect

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(temp)	1	0.003	0.0028	0.0051	0.9437
as.factor(conc)	3	76.338	25.4460	45.9647	9.652e-11 ***
Residuals	27	14.947	0.5536		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

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